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<b>(21) International Application Number:</b> PCT/US99/14749 <b>(22) International Filing Date:</b> 29 June 1999 (29.06.99)  <b>(30) Priority Data:</b> 60/094,407                      28 July 1998 (28.07.98)                      US  <b>(71) Applicant (for all designated States except US):</b> THE UNITED STATES OF AMERICA, represented by THE SECRETARY DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ZOON, Kathryn, C. [US/US]; 9709 Culver Street, Kensington, MD 20895 (US). HU, Renqiu [US/US]; 5021 Alta Vista Road, Bethesda, MD 20814 (US). BEKISZ, Joseph, B. [-/US]; 3921 Oglethorpe Street, Hyattsville, MD 20782 (US). HAYES, Mark, P. [US/US]; 1302 Hawthorne Road, Wilmington, NC 28403 (US).		<b>(74) Agent:</b> NOONAN, William, D.; Klarquist, Sparkman, Campbell, Leigh & Whinston, LLP, One World Trade Center, Suite 1600, 121 SW Salmon Street, Portland, OR 97204 (US).  <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> INTERFERON ALPHA HYBRIDS  <b>(57) Abstract</b>  Hybrid human interferon- $\alpha$ polypeptides, and the corresponding nucleic acid molecules, are disclosed. Pharmaceutical compositions comprising these peptides, and the use of these polypeptides to treat viral disease and regulate cell growth are also provided.		

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## INTERFERON ALPHA HYBRIDS

### FIELD OF THE INVENTION

This invention relates to human interferon- $\alpha$  hybrids and nucleic acid molecules that encode  
5 these hybrids.

### BACKGROUND OF THE INVENTION

Interferons are cytokines produced by a variety of eukaryotic cells upon exposure to certain environmental stimuli, including mitogens, endotoxins, double stranded RNA, and viral infection. In  
10 addition to having antiviral properties, interferons have been shown to affect a wide variety of cellular functions. These effects include inhibition of cell proliferation, immune regulatory functions and activation of multiple cellular genes. Interferons (IFNs) have been classified into four groups according to their chemical, immunological, and biological characteristics:  $\alpha$  (leukocyte),  $\beta$  (fibroblast),  $\gamma$ , and  $\omega$ . IFNs are further identified by the eukaryote in which they originated, with  
15 HuIFN indicating human interferon, for instance.

HuIFN- $\alpha$ s are encoded by a multigene family consisting of about 20 genes; each gene encodes a single subtype of the HuIFN- $\alpha$ . Amino acid sequence identity among IFN- $\alpha$  subtypes is generally 80-85% (Horisberger and Di Marco 1995). HuIFN- $\alpha$  polypeptides are produced by a number of human cell lines and human leukocyte cells after exposure to viruses or double-stranded  
20 RNA, or in transformed leukocyte cell lines (*e.g.*, lymphoblastoid lines).

IFN- $\alpha$ s act through interaction with cell-surface receptors and induce the expression, primarily at the transcriptional level, of a broad but specific set of cellular genes. Several IFN-induced gene products have been used as markers for the biological activity of interferons. These include, for instance, ISG15, ISG54, IRF1, GBP, and IP10.

25 Individual IFN- $\alpha$  subtypes have different biological activities. For instance, it was recognized early in interferon research that IFN- $\alpha$ 1 and IFN- $\alpha$ 2 have distinct target-cell specificities. IFN- $\alpha$ 2 shows high specific activity on bovine and human cells (similar to most HuIFN- $\alpha$ s), whereas IFN- $\alpha$ 1 shows high activity only on bovine cells.

Interferon activities were first characterized in relation to viral infections, and IFN- $\alpha$ s have  
30 proven to be remarkably effective antiviral agents. The current definition of IFN activity units is expressed in virological terms. There are many assays known to those skilled in the art that measure the degree of resistance of cells to viruses (McNeill, 1981). These assays generally can be categorized into three types: inhibition of cytopathic effect; virus plaque formation; and reduction of virus yield. Viral cytopathic effect assays measure the degree of protection induced in cell cultures pretreated with IFN and subsequently infected with viruses. Vesicular stomatitis virus, for instance,  
35 is an appropriate virus for use in such an assay. This type of assay is convenient for screening numerous different IFNs, as it can be performed in 96-well plates (Rubinstein *et al.*, 1981). Plaque-reduction assays measure the resistance of IFN-treated cell cultures to a plaque-forming virus (for

instance, measles virus). One benefit to this assay is that it allows precise measurement of a 50% reduction in plaque formation. Finally, virus yield assays measure the amount of virus released from cells during, for instance, a single growth cycle. Such assays are useful for testing the antiviral activity of IFNs against viruses that do not cause cytopathic effects, or that do not build plaques in target-cell cultures. The multiplicity of infection (moi) is an important factor to consider when using either plaque-reduction or virus-yield assays.

Other clinically important interferon characteristics are also easily assayed in the laboratory setting. One such characteristic is the ability of an interferon polypeptide to bind to specific cell-surface receptors. For instance, some IFN- $\alpha$ s exhibit different cell-surface properties compared to IFN- $\alpha$ 2b, the IFN most widely used in clinical trials. While IFN- $\alpha$ 2b is an effective antiviral agent, it causes significant adverse side effects. Interferons that exhibit distinct binding properties from IFN- $\alpha$ 2b may not cause the same adverse effects. Therefore, interferons that compete poorly with IFN- $\alpha$ 2b for binding sites on cells are of clinical interest. Competitive interferon binding assays are well known in the art (Hu *et al.*, 1993; Di Marco *et al.*, 1994). In general, such assays involve incubation of cell culture cells with a mixture of  $^{125}$ I-labeled IFN- $\alpha$ 2b and an unlabeled interferon of interest. Unbound interferon is then removed, and the amount of bound label (and by extension, bound  $^{125}$ I-labeled IFN- $\alpha$ 2b) is measured. By comparing the amount of label that binds to cells in the presence or absence of competing interferons, relative binding affinities can be calculated.

Another prominent effect of IFN- $\alpha$ s is their ability to inhibit cell growth, which is of major importance in determining anti-tumor action. Growth inhibition assays are well established, and usually depend on cell counts or uptake of tritiated thymidine ( $^3$ H]thymidine) or another radiolabel. The human lymphoblastoid Daudi cell line has proven to be extremely sensitive to IFN- $\alpha$ s, and it has been used to measure antiproliferative activity in many IFN- $\alpha$ s and derived hybrid polypeptides (Meister *et al.*, 1986). Use of this cell line has been facilitated by its ability to be grown in suspension cultures (Evinger and Pestka, 1981).

IFN- $\alpha$ s also exhibit many immunomodulatory activities (Zoon *et al.*, 1986).

Although IFNs were first discovered by virologists, their first clinical use (in 1979) was as therapeutic agents for myeloma (Joshua *et al.*, 1997). IFN- $\alpha$ s have since been shown to be efficacious against a myriad of diseases of viral, malignant, angiogenic, allergic, inflammatory, and fibrotic origin (Tilg, 1997). For instance, IFN- $\alpha$  is the only drug that is currently approved for treatment of hepatitis C in Europe and North America (Moussalli *et al.*, 1998), and is the treatment of choice for chronic acute hepatitis B and AIDS-related Kaposi's sarcoma. It has also proven efficacious in the treatment of metastatic renal carcinoma and chronic myeloid leukemia (Williams and Linch, 1997). Clinical uses of IFNs are reviewed in Gresser (1997) and Pfeffer (1997).

Standard recombinant techniques have become useful methods for the production and modification of IFN- $\alpha$  proteins (Streuli *et al.*, 1981; Horisberger and Di Marco 1995; Rehberg *et al.*, 1982; Meister *et al.*, 1986; Fidler *et al.*, 1987; Sperber *et al.*, 1993; Mitsui *et al.*, 1993; Muller *et al.*, 1994; and Zav'Yalov and Zav'Yalov 1997). One such recombinant modification is the formation of

hybrid IFN molecules. Hybrid IFNs contain fragments of two or more different interferon polypeptides, functionally fused together. The first IFN- $\alpha$  hybrids were designed to study molecular structure-function relationships. Much research has since been directed toward the production of hybrid IFNs that combine different biological properties of the parental proteins. Some hybrid IFNs display biological activity that is significantly different from that of both parent molecules (Horisberger and Di Marco 1995). For instance, certain early IFN- $\alpha$ /IFN- $\alpha$  hybrids acquired the novel property of very high activity on mouse cells (Streuli *et al.*, 1980; Rehberg *et al.*, 1982).

The techniques used by researchers to generate hybrid IFN polypeptides have evolved through time (Horisberger and Di Marco 1995). Early researchers took advantage of the presence of naturally occurring restriction endonuclease (RE) cleavage sites within IFN-encoding sequences to piece together homologous coding fragments. (See, for instance, U.S. Pat. No. 5,071,761 "Hybrid Interferons"). Though convenient, this was a limited method in that only so many of such pre-existing RE sites occurred in each IFN coding sequence. In addition, the location of each restriction site was fixed, making the possible combinations relatively small. More recently, researchers have used PCR amplification to create specific desired nucleic acid fragments, thereby gaining the ability to piece together new pieces of different IFNs (Horton *et al.*, 1989).

A number of U.S. patents discuss various hybrid IFNs, how to produce them, and how to use them to treat patients. Many such patents relate to inter-group (multi-class) hybrid IFNs, wherein portions of the final hybrid are taken from at least two different interferon classification groups (*e.g.*,  $\alpha$  and  $\beta$ ). For instance, U.S. Pat. No. 4,758,428 ("Multiclass hybrid interferons") describes the multi-class hybrid IFN HuIFN- $\alpha$ 1(1-73)/HuIFN- $\beta$ 1(74-166), and its use in pharmaceutical compositions to treat viral infections and tumorous growths in animal patients. Another such patent (U.S. Pat. No. 4,914,033 "Structure and properties of modified interferons") discloses the making of constructs that encode hybrid interferons comprising amino- and carboxy-terminal fragments of HuIFN- $\beta$  fused to an internal sequence (amino acid residues 36-46) of a HuIFN- $\alpha$ . This patent also discloses the purification of the encoded hybrid IFN polypeptide and its use in pharmaceutical formulations.

Intra-group hybrid interferons (*e.g.*,  $\alpha$ 1/ $\alpha$ 8 hybrids) have also been described. U.S. Pat. No. 5,071,761 ("Hybrid interferons") provides a good example of such intra-group hybrids. This patent discloses the construction, purification, use, and pharmaceutical preparation of various fusions hybrids between HuIFN- $\alpha$ 1 and HuIFN- $\alpha$ 8, where as many as four distinct IFN- $\alpha$  fragments have been used to construct the fusion. The construction, purification, and use of similar IFN- $\alpha$  hybrids to treat animal patients are disclosed in U.S. Pat. No. 5,137,720 ("Antiviral combination, and method of treatment").

It is to such engineered, recombinant intra-group hybrid interferon molecules that the present invention is directed.

## SUMMARY OF THE INVENTION

The present invention provides hybrid interferons constructed by combining portions of two or more interferon- $\alpha$ s, and mutant and mutant hybrid interferons constructed by point mutagenesis.

5 These interferon molecules have good antiviral and antiproliferative activities. Thus, they may be used clinically to treat viral infections (such as influenza, rabies, and hepatitis B) and tumors, including but not limited to osteogenic sarcoma, multiple myeloma, nodular, poorly differentiated lymphoma, leukemia, carcinoma, melanoma, and papilloma, as well to modulate the immune system.

Six of the hybrids provided by this invention are termed HY-1, HY-2, HY-3, HY-4, HY-5,  
10 and HY-6, and are composed as follows:

HY-1: IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-165);

HY-2: IFN- $\alpha$ 21a(1-95)/IFN- $\alpha$ 2c(96-165);

HY-3: IFN- $\alpha$ 2c(1-95)/IFN- $\alpha$ 21a(96-166);

15 HY-4: IFN- $\alpha$ -21a(1-75)/IFN- $\alpha$ 2c(76-81)/IFN- $\alpha$ 21a(82-95)/IFN- $\alpha$ 2c(96-165);

HY-5: IFN- $\alpha$ -21a(1-75)/IFN- $\alpha$ 21a(76-81)/IFN- $\alpha$ 2c(82-95)/IFN- $\alpha$ 2c(96-165);

HY-6: IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-95)/IFN- $\alpha$ 21a(96-166).

This nomenclature indicates that HY-1 is comprised of amino acids 1-75 of IFN- $\alpha$ 21a fused to amino  
20 acids 76-165 of IFN- $\alpha$ 2c; HY-2 is comprised of amino acids 1-95 of IFN- $\alpha$ 21a fused to amino acids 96-165 of IFN- $\alpha$ 2c; HY-3 is comprised of amino acids 1-95 of IFN- $\alpha$ 2c fused to amino acids 96-166 of IFN- $\alpha$ 21a; and so forth for the remaining mutants.

Further aspects of the invention include the hybrid IFNs HY-1, HY-2, HY-3, HY-4, HY-5,  
and HY-6 and nucleic acid molecules that encode these hybrid interferons. Also encompassed within  
25 the scope of the invention are recombinant vectors that comprise such a nucleic acid molecule. Such vectors can be transformed into various cells to gain expression of these hybrid interferons. Accordingly, the invention also encompasses a cell transformed with a recombinant vector comprising such a nucleic acid molecule.

While each of these hybrids has particular advantages for clinical use, HY-3 in particular  
30 shows striking antiproliferative activity. This activity is associated with the combination of the 76-95 region of IFN- $\alpha$ 2c and the 96-166 region of IFN- $\alpha$ 21a. Accordingly, another aspect of the invention comprises HY-3-like molecules comprising such IFN components. Such molecules may be represented as X-A-B wherein "X" comprises about amino acid residues 1-75 of any IFN- $\alpha$ , "A" comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c and "B" comprises about amino acid residues  
35 96-166 of IFN- $\alpha$ 21a. While precise numerical limitations for the size of these sub-regions are provided (e.g., about amino acid residues 76-95), one of ordinary skill in the art will appreciate that these biological molecules may be varied in exact size. In certain embodiments, such variations will

be no greater than plus or minus five amino acids from the specified termination points. Likewise, residues 81-90 or 81-95 of IFN- $\alpha$ 2c comprise about the same amino acid residues as residues 76-95.

Strong antiproliferative activity may also be obtained by combining about IFN- $\alpha$ 2c(76-95) with amino- and carboxy-regions of other IFNs. Because IFN- $\alpha$ s are either 165 or 166 amino acids in length, the full length of these hybrids will be generally either 165 or 166 residues, depending on what IFN- $\alpha$  the carboxy-region is taken from. In such hybrids, the amino- (about residues 1-75) and carboxy- (about residues 96-165/166) regions may be provided from a single IFN- $\alpha$ , or from two different IFN- $\alpha$ s. These hybrid IFN molecules may be represented as X-A-Y, wherein "X" comprises about amino acid residues 1-75 of any IFN- $\alpha$ , "A" comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and "Y" comprises about amino acid residues 96-165/166 of any IFN- $\alpha$ . Another aspect of the invention is a recombinant IFN hybrid protein comprising first, second, and third domains, wherein the first domain comprises the amino-region of an IFN- $\alpha$ , the second domain comprises the middle region of IFN $\alpha$ 2c (about residues 76-95), and the third domain comprises the carboxy-region of an IFN- $\alpha$ .

In certain embodiments of the invention, a shorter region of IFN- $\alpha$ 2c contained within the region from residue 76 to residue 95 will be sufficient to confer substantial antiproliferative activity on a hybrid interferon containing such a fragment. As above, such hybrid interferons will be generally either 165 or 166 residues, depending on what IFN- $\alpha$  the carboxy- region is taken from. The amino- and carboxy- terminal regions are provided from a single IFN- $\alpha$  or from two different IFN' $\alpha$ s. Such a hybrid interferon- $\alpha$  molecule with a short IFN- $\alpha$ 2c middle region may be represented as V-C-Y, wherein "V" comprises about amino acid residues 1-81 of an interferon- $\alpha$ , "C" comprises about amino acid residues 82-95 of IFN- $\alpha$ 2c, and "Y" comprises about amino acid residues 96-165/166 of an interferon- $\alpha$ .

In particular embodiments of the invention, the third domain of the protein comprises about amino acid residues 96-166 of IFN- $\alpha$ 21a. In these embodiments, the first domain of the protein comprises the amino-region of any IFN- $\alpha$ . Such a hybrid IFN can be represented generally as X-A-B, wherein "X" comprises about amino acid residues 1-75 of an interferon- $\alpha$ , "A" comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and "B" comprises about amino acid residues 96-166 of IFN- $\alpha$ 21a.

Hybrid interferon molecules according to the present invention can also contain more than three segments or domains of different parental interferons. Such multiple domains are taken from at least two different source or parental interferons, and may be taken from up to as many different interferon- $\alpha$ s as there are segments assembled to construct the hybrid. For instance, a four-domain hybrid interferon- $\alpha$  will be constructed from as few as two or as many as four different interferon- $\alpha$ s.

One four domain hybrid interferon- $\alpha$  molecule encompassed within the current invention can be designated M-N-O-P, wherein "M" comprises about amino acid residues 1-75 of interferon  $\alpha$ 21a, "N" comprises about amino acid residues 76 to 81 of interferon- $\alpha$ 2c, "O" comprises about

amino acid residues 82 to 95 of interferon- $\alpha$ 21a, and "P" comprises about amino acid residues 96 to 165 of interferon- $\alpha$ 2c. A representative four domain hybrid interferon- $\alpha$  of this type is HY-4.

The invention also provides nucleic acid molecules that encode any of the multi-domain hybrid IFN proteins disclosed herein, including those that can be represented generally as X-A-B, X-A-Y, V-C-Y, and M-N-O-P, as well as recombinant vectors that comprise such a nucleic acid molecule and cells transformed with such a vector.

One of ordinary skill in the art will also appreciate that minor modifications to the IFN- $\alpha$  sequences described herein may also be employed, such as amino acid substitutions, additions, and deletions, to create a mutant hybrid interferon- $\alpha$ . Thus, it is entirely possible that hybrid IFNs having greater than or fewer than 165/166 amino acids may be produced. Substitutions will typically be conservative in nature (e.g., one aliphatic amino acid for another), and such modifications will generally be designed not to have a significant effect on the biological properties of the hybrid IFN.

Also encompassed are purified or isolated interferon- $\alpha$ s (such as IFN- $\alpha$ 2d) that contain point mutations at either residue 86 or residue 90, thereby changing these residues to tyrosine. Such mutant interferon- $\alpha$ s may be mutant hybrid molecules, and such mutant hybrids can contain short or long segments of IFN- $\alpha$ 2c, IFN- $\alpha$ 21a, or both of these parental interferons. Specific representatives of these mutant hybrid interferons include SDM-1 and SDM-2. Additional mutations can be made to replace existing tyrosine residues at 86 or 90 with other amino acids; specific representatives of this type of mutant hybrid interferon are SDM-3, and SDM-4.

Further aspects of the invention include nucleic acid molecules that encode the mutant hybrid interferons as disclosed herein, and particularly SDM-1, SDM-2, SDM-3, and SDM-4. Recombinant vectors that comprise such a nucleic acid molecule are also encompassed. Such vectors can be transformed into various cells to gain expression of these mutant interferons. Accordingly, the invention also encompasses a cell transformed with a recombinant vector comprising such a nucleic acid molecule.

The invention further provides pharmaceutical compositions comprising a pharmaceutically acceptable vehicle or carrier and at least one hybrid IFN- $\alpha$  polypeptide as described above. Such hybrid IFN- $\alpha$ s include those generally represented as X-A-Y, as X-A-B, as V-C-Y, and as M-N-O-P, as well as the specific hybrids HY-1, HY-2, HY-3, HY-4, HY-5, and HY-6. Mutant hybrid IFN- $\alpha$ s (e.g., SDM-1, SDM-2, SDM-3, or SDM-4) may also be included in such pharmaceutical compositions, either singly, in combinations with other mutant hybrid interferons, or in combination with hybrids IFNs as listed above.

These pharmaceutical compositions can be administered to humans or other animals on whose cells they are effective, in various manners such as topically, orally, intravenously, intramuscularly, intraperitoneally, intranasally, intradermally, intrathecally, and subcutaneously. Accordingly, a further aspect of the invention is such a pharmaceutical composition that is an injectable composition.



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The invention also encompasses methods for treating a patient for a viral disease, comprising administering to the patient a therapeutically effective, viral disease-inhibiting amount of one or more hybrid or mutant hybrid interferon- $\alpha$ s as described above. One specific aspect of this invention is a method of treatment, wherein the hybrid interferon- $\alpha$  is administered to the patient by injection.

Another aspect of the invention encompasses methods for regulating cell growth in a patient, comprising administering to the patient a therapeutically effective, cell growth-regulating amount of one or more hybrid or mutant hybrid interferon- $\alpha$ s as described above. The cell growth regulated by this treatment may be, for instance, tumor cell growth. One specific aspect of this invention is a method of regulating cell growth, wherein the hybrid or mutant hybrid interferon- $\alpha$  is administered to the patient by injection.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the general PCR strategy used to construct interferon- $\alpha$  hybrids. Fig. 1(A) shows the strategy for construction of HY-1; Fig. 1(B) shows the strategy for construction of HY-2; and Fig 1(C), that for HY-3 construction.

Fig. 2 shows the antiproliferative effects of IFN- $\alpha$ 2c ( $\square$ ) and IFN- $\alpha$ 21a ( $\nabla$ ) compared to that of hybrid IFNs HY-1 ( $\Delta$ ), HY-2 (O) and HY-3 (\*). Panel A, Daudi cells, Panel B, WISH cells, and Panel C, primary human lymphocytes.

Fig. 3 shows the antiviral activities of IFN- $\alpha$ 2c and IFN- $\alpha$ 21a compared to that of hybrid IFNs HY-2 and HY-3 on primary human lymphocytes. Legend: a, No IFN; b, IFN- $\alpha$ 2c; c, IFN- $\alpha$ 21a; d, HY-2; e, HY-3.

Fig. 4 shows the competitive binding curves for  $^{125}$ I-labeled IFN- $\alpha$ 2b using native interferons IFN- $\alpha$ 2c ( $\square$ ) and IFN- $\alpha$ 21a ( $\nabla$ ), and hybrid interferons HY-1 ( $\Delta$ ), HY-2 (O) and HY-3 (\*) as competitors. Panel A, Daudi cells; Panel B, WISH cells.

Table 1 summarizes the results of antiproliferative, antiviral, and competitive binding activity assays using parental IFNs  $\alpha$ 2 and  $\alpha$ 21a, hybrids HY-1, HY-2, HY-3, HY-4, and HY-5, and interferon mutant hybrids SDM-1, SDM-2, SDM-3, and SDM-4. Antiproliferative activities are reported as the amount (ng/ml) of each IFN species needed to inhibit cell growth by 50%. N/D: No Data.

#### SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but it is understood that the complementary strand is included by any reference to the displayed strand.

SEQ ID NO: 1 shows the outside PCR primer used for synthesis of hybrids HY-1, HY-2, HY-3, HY-4, HY-5, and HY-6, and mutant hybrids SDM-1, SDM-3, SDM-3, and SDM-4. This primer contains an engineered BamHI restriction site, and was used as the upstream primer during IFN- $\alpha$ 21a (HY-1 and HY-2) and IFN- $\alpha$ 2c (HY-3) amplification. This primer was also used as the  
5 upstream primer for amplification of IFN- $\alpha$ 21a from cDNA.

SEQ ID NO: 2 shows the inside PCR primer used for synthesis of HY-1. This primer was used as the downstream primer during IFN- $\alpha$ 21a amplification.

SEQ ID NO: 3 shows the inside PCR primer used for synthesis of HY-1. This primer was used as the upstream primer during IFN- $\alpha$ 2c amplification.

10 SEQ ID NO: 4 shows the outside PCR primer used for synthesis of HY-1 and HY-2. This primer contains an engineered SphI restriction site, and was used as the downstream primer during IFN- $\alpha$ 2c amplification.

SEQ ID NO: 5 shows the inside PCR primer used for synthesis of HY-2 and HY-3. This primer was used as the downstream primer during IFN- $\alpha$ 21a amplification.

15 SEQ ID NO: 6 shows the inside PCR primer used for synthesis of HY-2 and HY-3. This primer was used as the downstream primer during IFN- $\alpha$ 21a amplification.

SEQ ID NO: 7 shows the outside PCR primer used for synthesis of HY-3. This primer contains the engineered SphI restriction site, and was used as the downstream primer during IFN- $\alpha$ 2c (HY-2) and IFN- $\alpha$ 21a (HY-3) amplification. This primer was also used as the downstream primer  
20 for amplification of IFN- $\alpha$ 21a from cDNA.

SEQ ID NO: 8 shows the DNA coding sequence and corresponding amino acid sequence of HY-1.

SEQ ID NO: 9 shows the amino acid sequence of HY-1.

25 SEQ ID NO: 10 shows the DNA coding sequence and corresponding amino acid sequence of HY-2.

SEQ ID NO: 11 shows the amino acid sequence of HY-2.

SEQ ID NO: 12 shows the DNA coding sequence and corresponding amino acid sequence of HY-3.

SEQ ID NO: 13 shows the amino acid sequence of HY-3.

30 SEQ ID NOs: 14 and 15 show the inside primers used for synthesis of HY-4.

SEQ ID NOs: 16 and 17 show the inside primers used for synthesis of HY-5

SEQ ID NOs: 18 and 19 show the inside primers used for synthesis of HY-6.

SEQ ID NOs: 20 and 21 show the inside primers used for synthesis of SDM-1.

SEQ ID NOs: 22 and 23 show the inside primers used for synthesis of SDM-2.

35 SEQ ID NOs: 24 and 25 show the inside primers used for synthesis of SDM-3.

SEQ ID NOs: 26 and 27 show the inside primers used for synthesis of SDM-4.

SEQ ID NO: 28 Shows the outside primer used with SEQ ID NO: 1 for synthesis of HY-4, HY-5, HY-6, SDM-1, SDM-2, SDM-3, and SDM-4.

SEQ ID NO: 29 shows the DNA coding sequence and corresponding amino acid sequence of HY-4.

5 SEQ ID NO: 30 shows the amino acid sequence of HY-4.

SEQ ID NO: 31 shows the DNA coding sequence and corresponding amino acid sequence of HY-5.

SEQ ID NO: 32 shows the amino acid sequence of HY-5.

10 SEQ ID NO: 33 shows the DNA coding sequence and corresponding amino acid sequence of HY-6.

SEQ ID NO: 34 shows the amino acid sequence of HY-6.

SEQ ID NO: 35 shows the DNA coding sequence and corresponding amino acid sequence of SDM-1.

SEQ ID NO: 36 shows the amino acid sequence of SDM-1.

15 SEQ ID NO: 37 shows the DNA coding sequence and corresponding amino acid sequence of SDM-2.

SEQ ID NO: 38 shows the amino acid sequence of SDM-2.

SEQ ID NO: 39 shows the DNA coding sequence and corresponding amino acid sequence of SDM-3.

20 SEQ ID NO: 40 shows the amino acid sequence of SDM-3.

SEQ ID NO: 41 shows the DNA coding sequence and corresponding amino acid sequence of SDM-4.

SEQ ID NO: 42 shows the amino acid sequence of SDM-4.

25 The amino acid sequences of the hybrid IFNs are depicted without leader sequences. Such leader sequences are typically present on IFNs produced in eukaryotic cells, but are generally cleaved off to produce the mature form of the protein. The nomenclature for IFNs used herein is based on the amino acid sequences of mature IFNs.

## 30 DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions and Abbreviations

#### A. Abbreviations

IFN: interferon

35 IFN- $\alpha$ : interferon- $\alpha$

HuIFN- $\alpha$ : human interferon- $\alpha$

IU: international units

MDBK: Madin-Darby bovine kidney cells

ATCC: American Type Culture Collection

*E. coli*: *Escherichia coli*

PHA: phytohemagglutinin

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

5 poly-DI-DC: polydeoxyinosine-deoxycytosine

RNase: ribonuclease

RE: restriction endonuclease

moi: multiplicity of infection

## 10 B. Definitions

Unless otherwise noted, technical terms are used according to conventional usage.

Definitions of common terms in molecular biology may be found in Lewin, *Genes V* published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert

15 A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8). The nomenclature for DNA bases and the three-letter code for amino acid residues, as set forth at 37 CFR § 1.822, are used herein. All interferon units are expressed with reference to the NIH human lymphoblastoid IFN standard Ga 23-901-532.

20 In order to facilitate review of the various embodiments of the invention, the following definitions of terms are provided. These definitions are not intended to limit such terms to a scope narrower than would be known to a person of ordinary skill in the field.

**Interferons:** A family of secreted polypeptides produced by a variety of eukaryotic cells upon exposure to various environmental stimuli, including virus infection or exposure to a mitogen. In addition to having antiviral properties, interferons have been shown to affect a wide variety of cellular functions. Interferons (IFNs) have been classified into four major groups according to their chemical, immunological, and biological characteristics:  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\omega$ . Each IFN is further identified by the eukaryote in which it originated, with HuIFN indicating human interferon. For the purposes of this disclosure, any interferon that lacks a specific eukaryote source designation is presumed to be that set of equivalent interferons from any source. In other words, IFN- $\alpha$ 2c would refer to the interferon- $\alpha$ 2c from any eukaryotic source, while HuIFN- $\alpha$ 2c refers specifically to human interferon- $\alpha$ 2c. Throughout this specification, the IFN nomenclature provided by Allen and Diaz (1996) is employed unless otherwise noted.

35 Interferon- $\alpha$  (IFN- $\alpha$ ) polypeptides are produced in, for instance, human leukocyte cells after exposure to viruses or double-stranded RNA, or in transformed leukocyte cell lines (e.g., lymphoblastoid lines). Most IFN- $\alpha$ s are non-glycosylated polypeptides of 165 or 166 amino acids, encoded for by a multigene family of at least 20 genes. The difference in length is due to an amino acid deletion at the 44<sup>th</sup> position in certain IFNs, for instance IFN- $\alpha$ 2c. Each gene (termed IFNA1,

IFNA2, etc.) encodes a single IFN- $\alpha$  polypeptide subtype (termed IFN- $\alpha$ 1, IFN- $\alpha$ 2, etc., respectively). Amino acid sequence identity among IFN- $\alpha$  subtypes is generally 80-85% (Horisberger and Di Marco 1995). Within each subtype, individual sequence variants (IFN species) are further denoted with an additional letter designation, *e.g.*, IFN- $\alpha$ 2a, IFN- $\alpha$ 2b, and IFN- $\alpha$ 2c. The sequence differences between these species are often very small (1-3 amino acids).

**Hybrid interferons:** Recombinant interferon molecules that combine various segments from parental interferon molecules. Hybrids may be constructed using portions of two (or more) IFNs from different IFN groups (*e.g.*, one segment from an IFN- $\alpha$  polypeptide and another segment from an IFN- $\beta$  polypeptide) (see, for instance, U.S. Pat. Nos. 4,758,428 "Multiclass hybrid interferons"; 4,914,033 "Structure and properties of modified interferons"; and 4,917,887 "Hybrid interferons, their use as pharmaceutical compositions and as intermediate products for the preparation of antibodies and the use thereof and processes for preparing them"). These are referred to as inter-group or multi-class hybrids. Alternatively, hybrids can be formed using portions of two different IFN species from one IFN group (*e.g.*, one segment from each of two IFN- $\alpha$  polypeptides) (see, for instance, U.S. Pat. Nos. 4,806,347 "Interferon combinations"; 4,892,743 "Novel hybrid interferon species"; 5,071,761 "Hybrid interferons"; 5,137,720 "Antiviral combination, and method of treatment"; and 5,609,868 "Pharmaceutical compositions comprising hybrid  $\alpha$ -interferon"). These are referred to as intra-group hybrids. The construction and properties of certain IFN- $\alpha$ /IFN- $\alpha$  hybrids has been reviewed (Horisberger and Di Marco 1995).

Herein, hybrid interferon protein nomenclature is used as proposed in Allen and Diaz (1996). For instance, the hybrid interferon fusion HY-1 is fully designated as IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-165), wherein the amino-terminal end of the polypeptide consists of amino acids 1-75 of IFN- $\alpha$ 21a and the carboxy-terminal end consists of amino acids 76-165 of IFN- $\alpha$ 2c. Hybrids HY-2 [IFN- $\alpha$ 21a(1-95)/IFN- $\alpha$ 2c(96-165)], HY-3 [IFN- $\alpha$ 2c(1-95)/IFN- $\alpha$ 21a(96-166)], HY-4 [IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-81)/IFN- $\alpha$ 21a(82-95)/IFN- $\alpha$ 2c(96-165)], and HY-6 [IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-95)/IFN- $\alpha$ 21a(96-166)] are designated similarly. The same terminology can be used where otherwise contiguous regions of the same parental interferon molecule are re-joined in a final construct, as is true in HY-5 [IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 21a(76-81)/IFN- $\alpha$ 2c(82-95)/IFN- $\alpha$ 2c(96-165)].

It is possible to refer to general classes of hybrid IFN molecules by designating domains within the protein that are of interest. For instance, data indicate that it is likely the carboxy-terminal portion (residues 76-166) of HY-3 that is important for the observed high antiproliferative activity. A class of HY-3-like molecules that contains this carboxy-terminal portion can be represented generally as X-A-B, wherein "X" comprises about amino acid residues 1-75 of an interferon- $\alpha$ , "A" comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and "B" comprises about amino acid residues 96-166 of IFN- $\alpha$ 21a.

One of ordinary skill in the art will appreciate that these elements may be combined to produce HY-3-like molecules without necessarily splicing the components in the same place. It is

possible to use shorter or longer fragments of IFN- $\alpha$ 2c, fused to correspondingly longer or shorter fragments of IFN- $\alpha$ 21a. In such instances, the middle element of IFN- $\alpha$ 2c used to construct the hybrid molecule comprises residues 76-96, 76-97 or 76-98, while the carboxy-terminal element of IFN- $\alpha$ 21a would correspondingly comprise residues 97-166, 98-166, or 99-166, respectively. Any component that is spliced within 5 amino acid residues of the residue specified comprises about the same region. For instance, amino acid residues 1-80 or 1-70 of IFN- $\alpha$ 2c comprise about the same amino acid residues as the component with residues 1-75. Likewise, residues 81-90 or 81-95 of IFN- $\alpha$ 2c comprise about the same amino acid residues as this component with residues 76-95.

Further, hybrid interferon molecules can be constructed in which the middle region is defined as being from a specific source, for instance residues 76-95 of IFN- $\alpha$ 2c, but the amino- and carboxy-regions can be chosen from any IFN- $\alpha$ . Because IFN- $\alpha$ s are either 165 or 166 amino acids in length, the full length of these hybrids will be either 165 or 166 residues, depending on which IFN- $\alpha$ s are used to construct the hybrid. In such hybrids, the amino- (about residues 1-75) and carboxy- (about residues 96-165/166) regions may be provided from any single IFN- $\alpha$ , or from two different IFN- $\alpha$ s. These hybrid IFN molecules may be represented as X-A-Y, wherein "X" comprises about amino acid residues 1-75 of any IFN- $\alpha$ , "A" comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and "Y" comprises about amino acid residues 96-165/166 of any IFN- $\alpha$ . As above, any component that is spliced within 5 amino acid residues of the residue specified comprises about the same region. For instance, residues 81-90 or 81-95 of IFN- $\alpha$ 2c, serving as the "A" component of this construct, comprise about the same amino acid residues as "A" with residues 76-95 of IFN- $\alpha$ 2c.

**Parenteral:** Administered outside of the intestine, e.g., not via the alimentary tract. Generally, parenteral formulations are those that will be administered through any possible mode except ingestion. This term especially refers to injections, whether administered intravenously, intrathecally, intramuscularly, intraperitoneally, or subcutaneously, and various surface applications including intranasal, intradermal, and topical application, for instance.

**Pharmaceutically acceptable carriers:** The pharmaceutically acceptable carriers useful in this invention are conventional. Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the hybrid interferons herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

**Injectable composition:** A pharmaceutically acceptable fluid composition comprising at least an active ingredient. The active ingredient is usually dissolved or suspended in a physiologically acceptable carrier, and the composition can additionally comprise minor amounts of one or more non-toxic auxiliary substances, such as emulsifying agents, preservatives, and pH buffering agents and the like. Such injectable compositions that are useful for use with the hybrid interferons of this invention are conventional; appropriate formulations are well known in the art, and examples may be found in U.S. Pat. 5,609,868 ("Pharmaceutical compositions comprising hybrid  $\alpha$ -interferon").

**Therapeutically effective amount of IFN- $\alpha$ :** A quantity of interferon- $\alpha$  sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to inhibit viral proliferation or to regulate cell, and more specifically tumor cell, proliferation. See, U.S. Pat. Nos. 4,089,400 ("Polypeptides and process for the production thereof") and 5,503,828 ("Alpha interferon composition and method for its production from human peripheral blood leukocytes") for general disclosure as to the amounts of IFN- $\alpha$  that have proven efficacious in clinical settings. The same dose levels as are used in conventional (non-hybrid) interferon therapy may be used with hybrid interferons. In general, a dose of about  $10^5$  to  $10^8$  IU will be appropriate and may be administered more than once, for example daily, during a course of treatment. However, the effective amount of hybrid IFN- $\alpha$  will be dependent on the subject being treated, the severity of the affliction, and the manner of administration of the interferon.

The hybrid interferons disclosed in the present invention have equal application in medical and veterinary settings. Therefore, the general term "subject being treated" is understood to include all animals that produce interferon polypeptides, including humans or other simians, dogs, cats, horses, and cows.

**Probes and primers:** A nucleic acid probe comprises an isolated nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, *e.g.*, in Sambrook *et al.*, (1989) and Ausubel *et al.*, (1987).

Primers are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length. Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand. The primer may be then extended along the target DNA strand through the use of a DNA polymerase enzyme. Primer pairs (one on either side of the target nucleic acid sequence) can be used for amplification of a nucleic acid sequence, *e.g.*, by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in Sambrook *et al.*, (1989), Ausubel *et al.*, (1987), and Innis *et al.*, (1990). PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose

such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with its length. Thus, for example, a primer comprising 20 consecutive nucleotides of one human IFN- $\alpha$  subtype cDNA or gene will anneal to a target sequence (e.g., a different human IFN- $\alpha$  subtype or an IFN- $\alpha$  from another species) with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers may be selected that comprise 20, 25, 30, 35, 40, 50 or more consecutive nucleotides of one IFN- $\alpha$  subtype cDNA or gene sequence.

**Vector:** A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

**Transformed:** A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, particle gun acceleration, and the like.

**Isolated:** An "isolated" biological component (such as a nucleic acid or protein) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA and RNA, and proteins. Thus, nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

**Purified:** The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified IFN- $\alpha$  preparation is one in which the interferon-alpha is more enriched than the protein is in its natural environment within a cell. Preferably, a preparation of IFN- $\alpha$  is purified such that the IFN- $\alpha$  represents at least 50% of the total protein content of the preparation.

**Operably linked:** A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

**Recombinant:** A recombinant nucleic acid is one that has a sequence that is not naturally occurring, or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or,



more commonly, by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques, or a combination of these techniques. Similarly, a recombinant protein is one that is encoded by a recombinant nucleic acid.

5    **II.    Construction of Hybrid and Mutant Hybrid Interferon- $\alpha$ s**

**A.    General approaches to hybrid interferon- $\alpha$  construction**

          The production of a number of hybrid IFNs has been reviewed by Horisberger and Di Marco  
10   (1995); this article provides a good overview of the process of construction of such molecules. Specific examples of methods for construction of hybrid interferons can be found, for example, in U.S. Pat. Nos. 4,892,743 ("Novel hybrid interferon species"); 5,071,761 ("Hybrid Interferons"); 4,758,428 ("Multiclass hybrid interferons"); and 4,716,217 ("Hybrid lymphoblastoid-leukocyte human interferons").

15           Generally, two procedures are used to create hybrid IFN- $\alpha$ s. First, some researchers have taken advantage of the presence of naturally occurring RE cleavage sites within IFN-encoding sequences to piece together homologous coding fragments. (See, for instance, U.S. Pat. No. 5,071,761 "Hybrid Interferons"). The second general procedure for construction of hybrid IFN- $\alpha$ s uses PCR amplification to create specific desired nucleic acid fragments, thereby gaining the  
20   potential to piece together new pieces of different IFNs (Horton *et al.*, 1989). It is this second technique that has been employed herein to generate novel and useful IFN- $\alpha$  hybrids.

**B.    Construction of parental interferon-bearing plasmids**

          A plasmid bearing the IFN- $\alpha$ 2c coding sequence (pBluescript/A2) was constructed as  
25   previously described (Hayes and Zoon, 1993).

          To construct a plasmid bearing the coding sequence of IFN- $\alpha$ 21a, a pair of oligonucleotides, SEQ ID NO: 1 and SEQ ID NO: 7, with BamHI and PstI restriction sites, were synthesized based on the cDNA coding region for mature human IFN- $\alpha$ 21a protein (Genentech, South San Francisco, CA). These were used as primers in a standard polymerase chain reaction (PCR) (Innis *et al.*, 1990), and  
30   the entire coding region for mature human IFN- $\alpha$ 21a protein amplified. The resulting products were cleaved with restriction endonucleases (REs) BamHI and PstI and cloned into the *E. coli* expression vector pQE30 (QIAGEN, Chatsworth, CA) cleaved with REs BamHI and PstI, to form pQE30/A21. The final construct was verified by DNA sequence analysis (Sanger *et al.*, 1977).

35           **C.    Construction of HY-1**

          The hybrid IFN cDNAs presented in this invention were constructed by PCR technology (Horton *et al.*, 1989). The procedure used to construct HY-1 is illustrated in Fig 1(A). In reaction one, primers 1 (SEQ ID NO: 1) and 2 (SEQ ID NO: 2) were used to amplify the amino-terminal portion of IFN- $\alpha$ 21a (encoding amino acids 1-75), using linearized pQE30/A21 as template. In

reaction two, primers 3 (SEQ ID NO: 3) and 4 (SEQ ID NO: 4) were used to amplify the carboxy-terminal portion of IFN- $\alpha$ 2c (encoding amino acids 76-165), using linearized pBluescript/A2 as template. Purified DNA fragments from the first pair of PCR reactions were then mixed as templates for the second round of PCR, and the fused sequence amplified using primers 1 (SEQ ID NO: 1) and 4 (SEQ ID NO: 4). This reaction generated the full-length HY-1 fusion coding sequence (SEQ ID NO: 8), which encodes IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-165) (SEQ ID NO: 9).

Purified DNA fragments from the second round of PCR amplification were digested with REs BamHI and SphI and ligated into pQE30. The final HY-1 construct (pHY-1) was verified by DNA sequence analysis (Sanger *et al.*, 1977). HY-1 has been submitted to GenBank for publication on or after July 7<sup>th</sup>, 1999, accession number AF085803.

#### D. Construction of HY-2

The procedure used to construct HY-2 is illustrated in Fig 1(B). In reaction one, primers 1 (SEQ ID NO: 1) and 5 (SEQ ID NO: 5) were used to amplify the amino-terminal portion of IFN- $\alpha$ 21a (encoding amino acids 1-95), using linearized pQE30/A21 as template. In reaction two, primers 6 (SEQ ID NO: 6) and 4 (SEQ ID NO: 4) were used to amplify the carboxy-terminal portion of IFN- $\alpha$ 2c (encoding amino acids 96-165), using linearized pBluescript/A2 as template. Purified DNA fragments from the first pair of PCR reactions were then mixed as templates for the second round of PCR, and the fused sequence amplified using primers 1 (SEQ ID NO: 1) and 4 (SEQ ID NO: 4). This reaction generated the full-length HY-2 fusion coding sequence (SEQ ID NO: 10), which encodes IFN- $\alpha$ 21a(1-95)/IFN- $\alpha$ 2c(96-165) (SEQ ID NO: 11).

Purified DNA fragments from the second round of PCR amplification were digested with BamHI and SphI and ligated into pQE30. The final HY-2 construct (pHY-2) was verified by DNA sequence analysis (Sanger *et al.*, 1977). HY-2 has been submitted to GenBank for publication on or after July 7<sup>th</sup>, 1999, accession number AF085804.

#### E. Construction of HY-3

The procedure used to construct HY-3 is illustrated in Fig 1(C). In reaction one, primers 1 (SEQ ID NO: 1) and 5 (SEQ ID NO: 5) were used to amplify the amino-terminal portion of IFN- $\alpha$ 2c (encoding amino acids 1-95), using linearized pQE30/A21 as template. In reaction two, primers 6 (SEQ ID NO: 6) and 7 (SEQ ID NO: 7) were used to amplify the carboxy-terminal portion of IFN- $\alpha$ 21a (encoding amino acids 96-166), using linearized pBluescript/A2 as template. Purified DNA fragments from the first pair of PCR reactions were then mixed as templates for the second round of PCR, and the fused sequence amplified using primers 1 (SEQ ID NO: 1) and 7 (SEQ ID NO: 7). This generated the full-length HY-3 fusion coding sequence (SEQ ID NO: 12), which encodes IFN- $\alpha$ 2c(1-95)/IFN- $\alpha$ 21a(96-166) (SEQ ID NO: 13).

Purified DNA fragments from the second round of PCR amplification was digested with BamHI and SphI and ligated into pQE30. The final HY-3 construct (pHY-3) was verified by DNA

sequence analysis (Sanger *et al.*, 1977). HY-3 has been submitted to GenBank for publication on or after July 7<sup>th</sup>, 1999, accession number AF085805.

#### F. Construction of HY-4, -5, and -6

5 Using methods essentially similar to those discussed above for HY-1, -2, and -3, three further hybrid interferon molecules were constructed which incorporate shorter internal segments of the parent interferons. HY-4 was constructed using HY-2 as a template, and incorporates the following  $\alpha$ -interferon sequences: IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-81)/IFN- $\alpha$ 21a(82-95)/INF- $\alpha$ 2c(96-165). The nucleotide sequence of HY-4 is depicted in SEQ ID NO: 29. Primers 14s and 14as (SEQ  
10 ID NO: 14 and 15) served as the inside primers for construction of this hybrid.

HY-5 was constructed using HY-2 as a template, and incorporates the following interferon sequences: IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 21a(76-81)/IFN- $\alpha$ 2c(82-95)/INF- $\alpha$ 2c(96-165). The nucleotide sequence of HY-5 is depicted in SEQ ID NO: 31. Primers 15s and 15as (SEQ ID NO: 16 and 17) served as the inside primers for construction of this hybrid.

15 HY-6 was constructed using HY-1 and parental IFN- $\alpha$ 21a as templates, and incorporates the following interferon sequences: IFN- $\alpha$ 21a(1-75)/IFN- $\alpha$ 2c(76-95)/INF- $\alpha$ 21a(96-166). ). The nucleotide sequence of HY-6 is depicted in SEQ ID NO: 33. Primers M291s and M219as (SEQ ID NO: 18 and 19) served as the inside primers for construction of this hybrid. Primers 2\* and 1 (SEQ ID NO: 28 and 1) served as the outside primers for production of all three of these hybrids.

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#### G. Construction of mutant hybrid interferon- $\alpha$ s SDM-1, SDM-2, SDM-3, and SDM-4

In addition to the production of hybrid interferons from native sequences, it is also possible  
25 to construct hybrids that have specific sequence mutations at specific nucleotide and/or amino acid residues. As an example of this, four mutant interferon hybrids were constructed using methods essentially similar to those used above to construct the base hybrids. Mutations in specific amino acids were introduced into these mutant hybrids by incorporating desired nucleotide changes into the primers used for amplification of the relevant hybrid sequences.

30 SDM-1 was constructed using HY-4 as the template, and integrates a single amino acid mutation at residue 86, which changes the serine found in the IFN- $\alpha$ 21a sequence to a tyrosine. The nucleotide sequence of SDM-1 is depicted in SEQ ID NO: 35. Primers SDM1s and SDM1as (SEQ ID NO: 20 and 21) served as the inside primers for construction of this mutant.

SDM-2 was constructed using HY-4 as the template, and integrates a single amino acid  
35 mutation at residue 90, which changes the asparagine found in the IFN- $\alpha$ 21a sequence to a tyrosine. The nucleotide sequence of SDM-2 is depicted in SEQ ID NO: 37. Primers SDM2s and SDM2as (SEQ ID NO: 22 and 23) served as the inside primers for this mutant.

SDM-3 was constructed using HY-5 as the template, and integrates a single amino acid mutation at residue 86, which changes the tyrosine found in the IFN- $\alpha$ 2c sequence to a serine. The

nucleotide sequence of SDM-3 is depicted in SEQ ID NO: 39. Primers SDM3s and SDM3as (SEQ ID NO: 24 and 25) served as the inside primers for this mutant.

SDM-4 was constructed using HY-5 as the template, and integrates a single amino acid mutation at residue 90, which changes the tyrosine found in the IFN- $\alpha$ 2c sequence to an asparagine.

- 5 The nucleotide sequence of SDM-4 is depicted in SEQ ID NO: 41. Primers SDM4s and SDM4as (SEQ ID NO: 26 and 27) served as the inside primers for this mutant. Primers 2\* and 1 (SEQ ID NO: 28 and 1) served as the outside primers for production of all four mutants.

10 **III. Expression and Purification of Hybrid and Mutant Hybrid Interferons**

**A. Expression of IFN- $\alpha$  hybrids and mutants**

- The following method of expression of hybrid and mutant interferons is provided merely by way of example. One skilled in the art will understand that there are myriad ways to express a recombinant protein such that it can be subsequently purified. See, for instance, U.S. Pat. No. 15 5,089,400 ("Polypeptides and process for the production thereof"). In general, an expression vector carrying the nucleic acid sequence that encodes the desired protein will be transformed into a microorganism for expression. Such microorganisms can be prokaryotic (bacteria) or eukaryotic (e.g., yeast). One appropriate species of bacteria is *Escherichia coli* (*E. coli*), which has been used 20 extensively as a laboratory experimental expression system. A eukaryotic expression system will be preferred where the protein of interest requires eukaryote-specific post-translational modifications such as glycosylation.

- The expression vector can include a sequence encoding a targeting peptide, positioned in such a way as to be fused to the coding sequence of the IFN. This allows the hybrid IFN to be 25 targeted to specific locations. In a prokaryotic expression system, a signal sequence can be used to secrete the newly synthesized hybrid protein. In a eukaryotic expression system, the targeting peptide would specify targeting of the hybrid protein to one or more specific sub-cellular compartments, or to be secreted from the cell, depending on which peptide is chosen. One such appropriate targeting peptide is the native IFN signal peptide, which would direct the hybrid IFN to be secreted from 30 eukaryotic cells.

- Vectors suitable for stable transformation of bacterial cells are well known. Typically, such vectors include a multiple-cloning site suitable for inserting a cloned nucleic acid molecule, such that it will be under the transcriptional control of 5' and 3' regulatory sequences. In addition, transformation vectors include one or more selectable markers; for bacterial transformation this is 35 often an antibiotic resistance gene. Such transformation vectors typically also contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, and a transcription termination site, each functionally arranged in relation to the multiple-cloning site. For production of large amounts of recombinant proteins, an inducible promoter is preferred. This

permits selective production of the recombinant protein, and allows both higher levels of production than constitutive promoters, and enables the production of recombinant proteins that may be toxic to the expressing cell if expressed constitutively.

In addition to these general guidelines, protein expression/purification kits have been produced commercially. See, for instance, the QIAexpress™ expression system from QIAGEN (Chatsworth, CA) and various expression systems provided by INVITROGEN (Carlsbad, CA). Depending on the details provided by the manufactures, such kits can be used for production and purification of the disclosed hybrid interferons.

The following procedure can be used to express hybrid interferons as disclosed herein.

Plasmid DNA molecules carrying parental interferons IFNA2 (pBluescript/A2) and IFNA21 (pQE30/A21), and hybrid interferons HY-2 (pHY-2) and HY-3 (pHY-3) are transformed into *E. coli* strain SG13009 [pREP4] (QIAGEN, Chatsworth, CA). pHY-1 plasmid DNA is transformed into *E. coli* strain DH5 $\alpha$ F'IQ (Gibco BRL, Gaithersburg, MD). Bacteria are grown overnight in LB broth containing 100  $\mu$ g/ml ampicillin (pHY-1) or 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml kanamycin (pHY-2, pHY-3, pBluescript/A2 and pQE30/A21) in a 37°C shaker incubator. The cultures are diluted 1:50 in LB Broth containing the appropriate antibiotic(s) and incubated at 37°C with shaking, to a cell density of 0.8-0.9 A<sub>600</sub>. Protein expression is induced by the addition of 2 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG). The bacteria are then incubated at 30°C for 4-5 hours, after which cells were harvested and lysed by sonication. Each cell lysate is clarified by centrifugation at 10,000 x g for 30 minutes at 4°C. The resultant supernatants are used for subsequent purification of IFN polypeptides.

#### B. Purification of interferon hybrids and mutants

One skilled in the art will understand that there are myriad ways to purify recombinant interferon polypeptides. Typical methods of protein purification may be used to purify the disclosed interferons. Such methods include, for instance, monoclonal antibody affinity chromatography and isolation of insoluble protein inclusion bodies after over production. In addition, purification affinity-tags, for instance a hexa-histidine sequence, may be recombinantly fused to the protein and used to facilitate polypeptide purification. For further examples of purification of interferons, see U.S. Pat. No. 5,089,400 ("Polypeptides and process for the production thereof") and Zoon *et al.*, (1992).

In addition to the general protein purification procedures, certain modifications specific to interferon purification of may be beneficial. See for instance U.S. Pat. No. 5,593,667 ("Recombinant immune interferon having an intact carboxyl terminus"), disclosing IFN extraction techniques that overcome certain difficulties associated with degradation of the carboxy-terminal region of interferons during extraction and purification.

Commercially produced protein expression/purification kits provide tailored protocols for the purification of proteins made using each system. See, for instance, the QIAexpress™ expression system from QIAGEN (Chatsworth, CA) and various expression systems provided by INVITROGEN

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(Carlsbad, CA). Where a commercial kit is employed to produce the hybrid interferons, the manufacturer's purification protocol is a preferred protocol for purification of that hybrid. For instance, proteins expressed with an amino-terminal hexa-histidine tag can be purified by binding to nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrix (*The QIAexpressionist*,  
 5 QIAGEN, 1997)

By way of example only, the following procedure can be used to purify hybrid interferons. Expression of parental interferons IFN- $\alpha$ 2c and IFN- $\alpha$ 21a, and three IFN hybrids (HY-1, -2, and -3) is obtained in *E. coli* using the QIAexpress™ expression system plasmid pQE30. IFN polypeptide purification is first performed by Ni-NTA-Agarose resin metal-affinity chromatography (*The*  
 10 *QIAexpressionist*, QIAGEN, 1997; Janknecht *et al.*, 1991). The specific antiviral activity of this partially purified material ranges from  $3 \times 10^6$  IU/mg protein to  $4.5 \times 10^6$  IU/mg protein on Madin-Darby bovine kidney (MDBK) cells (ATCC #: CCL-22). To attain higher specific activity, the IFN- $\alpha$ s may optionally be further purified by monoclonal antibody affinity chromatography (*e.g.*, 4F2, NK2) (Zoon *et al.*, 1992). Final specific activities of each IFN species are shown in Table 1.  
 15 Generally, activities range from  $2 \times 10^8$  IU/mg protein to  $3.7 \times 10^8$  IU/mg protein on MDBK cells and from  $0.1 \times 10^8$  IU/mg protein to  $1.9 \times 10^8$  IU/mg protein on WISH cells (ATCC #: CCL-25). Purified recombinant protein concentrations are determined using the Coomassie Plus protein assay (PIERCE, Rockford, IL). Purity of the recombinant IFN- $\alpha$ s can be assessed by SDS-PAGE and HPLC analysis.

Similar procedures can be used to produce and purify the interferon hybrids HY-4, -5, and -  
 20 6, as well as mutant interferon hybrids SDM-1, -2, -3, and -4.

#### IV. Activity of Hybrid and Mutant Hybrid Interferons

##### A. Antiproliferative activity

25 The antiproliferative activities of several purified IFN- $\alpha$  hybrids and mutant hybrids were compared to parental interferons IFN- $\alpha$ 21a and IFN- $\alpha$ 2c. The ability of the IFN- $\alpha$  hybrids, mutants and both parents to inhibit the growth of Daudi, WISH and primary human lymphocyte cells was compared.

Human Daudi cells that are sensitive to IFN were obtained from Dr. P. Grimley, Dept. of  
 30 Pathology, USUHS, Bethesda MD. Cells were grown in suspension using RPMI 1640 with 10% fetal calf serum (FCS), 2 mM glutamine and 0.2% gentamicin. WISH cells as above were grown as monolayer cultures using Eagle's minimal essential medium supplemented with 10% FCS and gentamicin (50  $\mu$ g/ml). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All cultures were free of mycoplasma.

35 Antiproliferative assays on Daudi cells were performed as previously described (Hu *et al.*, 1993). The assays on WISH cells were performed by incubating the cells with various IFN- $\alpha$ s at concentrations ranging from 0.0003 ng/ml to 300 ng/ml for 72 hours at 37°C. A 50  $\mu$ l aliquot of 2 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added into each

well and incubated for 4 hours at 37°C. Then, 10% SDS in 0.01 N HCl (250 µl) was added to each well and incubated overnight at 37°C. The OD<sub>570</sub> of each well was determined, and the percentage of growth inhibition was calculated by comparing control (untreated) cultures with the IFN-treated cultures.

5           Primary human lymphocytes were treated with phytohemagglutinin (PHA) (Promega) at 1 µg/ml overnight, and the resultant PHA blasts treated for 72 hours with the various IFNs at the concentrations ranging from 0 to 1000 ng/ml. Percent inhibition of proliferation was calculated from direct cell counts, performed by Coulter counter analysis. Results are shown in Fig. 2.

10           The concentrations of IFN-αs that inhibited Daudi and WISH cell growth by 50% are shown in Table 1. HY-3 exhibited a higher antiproliferative activity than parental interferons IFN-α2c and IFN-α21a and the other hybrids on Daudi, WISH and primary human lymphocyte cells. In comparison, hybrids HY-2 and HY-4 have lower antiproliferative activities than the other hybrids or either of the parental IFN-αs on all these cells. HY-2 displays a 10,000 fold decrease in antiproliferative activity compared to HY-3 on Daudi cells and greater than a 1000 fold decrease on  
15           WISH and primary human cells. The hybrid HY-1 has a two- to eight-fold greater antiproliferative activity than HY-2 on Daudi and WISH cells. An intermediate level of antiproliferative activity on Daudi cells was found in the hybrid interferon HY-5 and the two mutant interferon hybrids SDM-1 and SDM-2.

#### 20           B.       Antiviral Activity

          The antiviral activities of purified IFN-α hybrids HY-1 through HY-5 and mutant hybrid interferons SDM-1 through SDM-4 were compared to that of parental interferons IFN-α21a and IFN-α2c. MDBK cells (ATCC, Manassas, VA; ATCC #: CCL-22) were prepared and maintained as described (Zoon *et al.*, 1992). WISH cells were prepared and maintained as above.

25           Antiviral activity was determined as previously described using MDBK cells and WISH cells (Zoon *et al.*, 1992). All IFN units are expressed with reference to the NIH human lymphoblastoid IFN standard Ga 23-901-532.

          The specific antiviral activities on MDBK and WISH cells are shown in Table 1. The antiviral specific activities of the five hybrids and four mutant hybrids are similar to each other and to  
30           IFN-α2c and IFN-α21a on MDBK cells (2.0x10<sup>8</sup> IU/ mg to 5.0x10<sup>8</sup> IU/mg). The specific activities of HY-1 and HY-2 are seven-fold lower than that of HY-3 on WISH cells.

          The Edmonston strain of measles virus (low passage, human embryonic kidney 7, VERO 5) (Albrecht *et al.*, 1981) was plaque-purified and used to infect 1 x 10<sup>6</sup> primary human lymphocytes that had been primed for expansion with phytohemagglutinin (PHA). Primary human lymphocytes  
35           were obtained from normal donors by centrifugal elutriation after Ficoll-Hypaque sedimentation (Lymphocyte Separation Medium (LSM) Package Insert, ORGANON TEKNIKA, Durham, NC). 1 x10<sup>8</sup> cells were resuspended in RPMI-1640 media supplemented with 10% FCS and fungizone (containing penicillin, streptomycin, and amphotericin B) with or without with 100 ng/ml of parental

or hybrid interferon for 24 hours prior to infection. These cells were then either infected with measles virus at moi of 0.1-1.6 TCID<sub>50</sub>/ml, or mock infected with virus-free medium, and harvested 72 hours post infection. Supernatants were titrated on VERO cell monolayers. Measles virus cytopathic effect was evaluated microscopically after 6 days and confirmed by staining with crystal violet. Figure 3 shows the results from lymphocytes from two donors. All experiments were preformed in triplicate and the results are expressed as percent of control; 100 percent for donors 1 and 2 were 4.95 and 5.7 TCID<sub>50</sub> log<sub>10</sub>, respectively.

### C. Interferon Binding

Interferon binding assays were performed as previously described on human Daudi (Hu *et al.*, 1993) and WISH (Zoon *et al.*, 1982) cells. Human IFN- $\alpha$ 2b was obtained from Schering Corp. (Kenilworth, NJ.), and has an antiviral specific activity of  $2 \times 10^8$  IU/mg protein. IFN- $\alpha$ 2b was labeled with <sup>125</sup>I-Bolton-Hunter reagent (Amersham, Arlington Heights, IL) as previously described (Hu *et al.*, 1993).

Fig. 4 shows the competitive binding curves for <sup>125</sup>I-labeled IFN- $\alpha$ 2b using IFN- $\alpha$ 2c, IFN- $\alpha$ 21a and the three hybrids (HY-1, HY-2, HY-3) as competitors on Daudi (Panel A) and WISH (Panel B) cells, respectively. The IFN- $\alpha$ 2c parent and the hybrid HY-3 compete very well for the <sup>125</sup>I-IFN- $\alpha$ 2b binding site on Daudi and WISH cells, while hybrids HY-1 and HY-2, like IFN- $\alpha$ 21a, compete poorly for the <sup>125</sup>I-IFN- $\alpha$ 2b binding site on Daudi cells. These results are summarized in Table 1.

### V. Production of Hybrid Interferon Variants

Of the hybrid IFN- $\alpha$ s herein described, HY-3 has by far the highest antiproliferative activity, exhibiting 1000-10,000-fold higher activity than HY-2 and HY-4. A possible explanation for this high activity is the existence of a domain affecting the antiproliferative activity within the carboxy-region (about residues 76-166) of HY-3. This region comprises a middle element of IFN- $\alpha$ 2c (about residues 76-95) fused to the carboxy-terminal element of IFN- $\alpha$ 21a (about residues 96-166). One of skill in the art will appreciate that these elements may be combined to produce HY-3-like molecules without necessarily splicing the components in the same place. It might be possible to use shorter or longer fragments of IFN- $\alpha$ 2c, fused to correspondingly longer or shorter fragments of IFN- $\alpha$ 21a. For instance, the middle element of IFN- $\alpha$ 2c might comprise residues 76-96, 76-97 or 76-98, while the carboxy-terminal element of IFN- $\alpha$ 21a would correspondingly comprise residues 97-166, 98-166, or 99-166, respectively. Any component that is spliced within 5 amino acid residues of the residue specified comprises about the same region. For instance, amino acid residues 1-80 or 1-70 of IFN- $\alpha$ 2c comprise about the same amino acid residues as the component with residues 1-75.

Although in the HY-3 fusion the amino-terminal element of the hybrid comprises amino acid residues 1-75 of IFN- $\alpha$ 2c, one skilled in the art will appreciate that other IFN- $\alpha$ s could be used



to provide this element. For instance, a HY-3-like polypeptide could be constructed that comprised residues 1-75 of IFN- $\alpha$ 21a fused to amino acid residues 76-166 of HY-3. Beyond this, it would be possible to use the amino-terminal element (residues 1-75) of any IFN- $\alpha$  (e.g., IFN- $\alpha$ 1, -2, -3, -4, etc.). The designation for a HY-3-like fusion of this type, wherein the amino-terminal 1-75 region  
5 comprises amino acids chosen from any IFN- $\alpha$  species, is IFN- $\alpha$ X(1-75)/IFN- $\alpha$ 2c(76-95)/IFN- $\alpha$ 21a(96-166), wherein "IFN- $\alpha$ X" designates any IFN- $\alpha$ , including but not limited to IFN- $\alpha$ 2c and IFN- $\alpha$ 21a. Alternately, such a HY-3-like molecule can be referred to generally as X-A-B, wherein "X" comprises about amino acid residues 1-75 of an interferon- $\alpha$ , "A" comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and "B" comprises about amino acid residues 96-166 of IFN- $\alpha$ 21a. As  
10 above, it can be appreciated also that these elements may be spliced in different places. For instance, the amino-terminal element may comprise residues 1-74 or 1-73, fused to amino acid residues 75-166 or 74-166 of HY-3, respectively. Amino-terminal fragments shorter than these could also be employed, with correspondingly longer middle regions.

It is further possible that the element that confers increased antiproliferative activity is found  
15 wholly within the middle element of IFN- $\alpha$ 2c (about residues 76-95). This is supported by the finding that HY-1 has higher antiproliferative activity than HY-2; the sole difference between these two hybrid interferons is the middle element. One skilled in the art will therefore appreciate that another HY-3-like polypeptide could be constructed which comprises an amino-terminal element (about residues 1-75) of any IFN- $\alpha$ , fused to the middle element (about residues 76-95) of IFN- $\alpha$ 2c,  
20 further fused to the carboxy-terminal element (about residues 96-165/166) of any IFN- $\alpha$ . Because IFN- $\alpha$ s are either 165 or 166 amino acids in length, the full length of these hybrids will be either 165 or 166 residues, depending on which IFN- $\alpha$ s are used to construct the hybrid. In such hybrids, the amino- (about residues 1-75) and carboxy- (about residues 96-165/166) regions may be provided from any single IFN- $\alpha$ , or from two different IFN- $\alpha$ s. These hybrid IFN molecules are represented  
25 as X-A-Y, wherein "X" comprises about amino acid residues 1-75 of any IFN- $\alpha$ , "A" comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and "Y" comprises about amino acid residues 96-165/166 of any IFN- $\alpha$ . In addition, as discussed above, the amino- and carboxy- regions may be shorter than those specified herein, for instance amino-regions of 1-74 or 1-73 residues, or carboxy-regions of 97-165/166 or 96-165/166. The corresponding middle region of IFN- $\alpha$ 2c will vary  
30 correspondingly in these latter hybrid molecules.

Shorter segments of the IFN- $\alpha$ 2c middle region, for instance residues 82-95, are sufficient to confer a substantial portion of the antiproliferative activity found in the HY-3 hybrid. This is evidenced by the high antiproliferative activity of HY-4 (SEQ ID NO: 30). In hybrid constructs using this shorter region of IFN- $\alpha$ 2c, the amino- (about residues 1-81) and carboxy- (about residues  
35 96-165/166) regions may be provided from any single IFN- $\alpha$ , or from two different IFN- $\alpha$ s, and as above will be generally either 165 or 166 residues, depending on what IFN- $\alpha$  the carboxy-region is taken from. Such a hybrid interferon- $\alpha$  molecule with a short IFN- $\alpha$ 2c middle region may be

represented as V-C-Y, wherein "V" comprises about amino acid residues 1-81 of an interferon- $\alpha$ , "C" comprises about amino acid residues 82-95 of IFN- $\alpha$ 2c, and "Y" comprises about amino acid residues 96-165/166 of an interferon- $\alpha$ .

More than three segments or domains of different parental interferons can be used to construct the hybrid IFN- $\alpha$ s of this invention. Such multiple domains are taken from at least two different source or parental interferons, and can be taken from up to as many different interferons as there are fragments assembled to construct the hybrid. A four-domain hybrid interferon- $\alpha$  therefore will be constructed from as few as two or as many as four different interferon- $\alpha$ s. The total length of these constructs will depend on the length(s) of the constituent parental interferons used.

One four domain hybrid interferon- $\alpha$  molecule may be represented as M-N-O-P, wherein "M" comprises about amino acid residues 1-75 of interferon  $\alpha$ 21a, "N" comprises about amino acid residues 76 to 81 of interferon- $\alpha$ 2c, "O" comprises about amino acid residues 82 to 95 of interferon- $\alpha$ 21a, and "P" comprises about amino acid residues 96 to 165 of interferon- $\alpha$ 2c. A representative four domain hybrid interferon of this type is HY-4.

At least a substantial portion of the antiproliferative activity found in HY-3 and the related hybrid and mutant interferons is linked to the presence of either or both of the tyrosine residues found at positions 86 and 90 in the fusion proteins. With the provision herein of the information that these residues are important in interferon biological activity, and specifically antiproliferative activity, this invention also encompasses mutant interferon- $\alpha$ s and mutant hybrid interferon- $\alpha$ s that contain point mutations at either residue 86 or residue 90, thereby changing these residues either to or from a tyrosine. Such point mutations can be introduced into interferon- $\alpha$ s and hybrid interferons through any available mutagenesis techniques, including but not limited to site-directed and PCR mediated mutagenesis. Mutant hybrid interferon polypeptides can for instance contain short or long segments of IFN- $\alpha$ 2c, IFN- $\alpha$ 21a, or both of these parental interferons. Specific representatives of these mutant hybrid interferons include SDM-1, SDM-2, SDM-3, and SDM-4.

#### **VI. Incorporation of Hybrid and Mutant IFN- $\alpha$ s into Pharmaceutical Compositions**

Pharmaceutical compositions that comprise at least one hybrid or mutant hybrid interferon as described herein as an active ingredient will normally be formulated with an appropriate solid or liquid carrier, depending upon the particular mode of administration chosen. The pharmaceutically acceptable carriers and excipients useful in this invention are conventional. For instance, parenteral formulations usually comprise injectable fluids that are pharmaceutically and physiologically acceptable fluid vehicles such as water, physiological saline, other balanced salt solutions, aqueous dextrose, glycerol or the like. Excipients that can be included are, for instance, other proteins, such as human serum albumin or plasma preparations. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Other medicinal and pharmaceutical agents, including non-hybrid interferons, also may be included.

The dosage form of the pharmaceutical composition will be determined by the mode of administration chosen. For instance, in addition to injectable fluids, topical and oral formulations can be employed. Topical preparations can include eye drops, ointments, sprays and the like. Oral formulations may be liquid (e.g., syrups, solutions or suspensions), or solid (e.g., powders, pills, tablets, or capsules). For solid compositions, conventional non-toxic solid carriers can include pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art; for example, see Remington's Pharmaceutical Sciences, E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975).

The pharmaceutical compositions that comprise hybrid interferon polypeptide will preferably be formulated in unit dosage form, suitable for individual administration of precise dosages. One possible unit dosage contains approximately 100 µg of protein. The amount of active compound administered will be dependent on the subject being treated, the severity of the affliction, and the manner of administration, and is best left to the judgment of the prescribing clinician. Within these bounds, the formulation to be administered will contain a quantity of the active component(s) in an amount effective to achieve the desired effect in the subject being treated.

The herein disclosed hybrid IFNs can also be administered to a patient using other acceptable delivery systems, including liposome-mediated delivery systems as disclosed in WO 96/17596 ("Liposomal interferon hybrid compositions").

The serum half-life of the administered hybrid IFN polypeptide can be extended in various ways, for instance, through formation of a complex with a monoclonal antibody. Such an antibody is usually directed to the hybrid IFN polypeptide at a site that does not materially impair its therapeutic activity (U.S. Pat. 5,055,289 "Interferon antibody compositions having an extended serum half-life"). Alternately, interferons can be conjugated to non-antigenic polymers, such as polyethylene glycol or related polyalkylene glycol moieties, to increase their serum persistence. See, for instance, Nieforth *et al.*, (1996) and U.S. Pat. Nos. 5,681,811 ("Conjugation-stabilized therapeutic agent compositions, delivery and diagnostic formulations comprising same, and method of making and using same"); 5,711,944 ("Interferon polymer conjugates"); and 5,738,846 ("Interferon polymer conjugates and process for preparing the same").

#### VII. Clinical Usage of Hybrid and Mutant Hybrid Interferons

The cell growth-regulating activity exhibited by the disclosed hybrid interferons makes these hybrids useful for treating tumors and cancers such as osteogenic sarcoma; multiple myeloma; Hodgkin's disease; nodular, poorly differentiated lymphoma; acute lymphocytic leukemia; acute myeloid leukemia; breast carcinoma; melanoma; papilloma; and nasopharyngeal carcinoma. In addition, the antiviral activity exhibited makes the disclosed hybrid interferons useful for treating viral infections in human and other animal patients. Possibly susceptible virus infections include, but

are not limited to, encephalomyocarditis virus infection, influenza and other respiratory tract virus infections, rabies and other viral zoonoses, and arbovirus infections, as well as herpes simplex keratitis, acute hemorrhagic conjunctivitis, varicella zoster, and hepatitis B and C.

The hybrid and mutant hybrid interferons of this invention may be administered to humans,  
5 or other animals on whose cells they are effective, in various manners such as topically, orally, intravenously, intramuscularly, intraperitoneally, intranasally, intradermally, intrathecally, and subcutaneously. Administration of hybrid interferon composition is indicated for patients with malignancies or neoplasms, whether or not immunosuppressed, or those patients requiring immunomodulation, or for antiviral treatment. The particular mode of administration and the dosage  
10 regimen will be selected by the attending clinician, taking into account the particulars of the case (e.g., the patient, the disease, and the disease state involved). For instance, tumor or cancer treatment typically involves daily or multi-daily doses of hybrid interferon over a period of months or even years. In contrast, viral infections are usually treated by daily doses of hybrid IFN over a few days to weeks. The same dose levels as are used in conventional (non-hybrid) interferon therapy may be  
15 used. See U.S. Pat. Nos. 4,089,400 ("Polypeptides and process for the production thereof") and 5,503,828 ("Alpha interferon composition and method for its production from human peripheral blood leukocytes") for general disclosure as to the amounts of IFN- $\alpha$  that have proven efficacious in clinical settings. In general, approximately  $10^5$  to  $10^8$  IU will be appropriate.

In addition to their individual use, a hybrid and mutant hybrid interferon as disclosed in the  
20 current invention may be combined with or used in association with other chemotherapeutic or chemopreventive agents for providing therapy against neoplasms or other conditions against which it is effective. See, for instance, U.S. Pat. No. 4,805,347 ("Interferon combinations"), which discloses various compositions and methods for treating tumors and viruses in humans by administering a combination of IFN- $\alpha$  and an IFN- $\alpha 2$ /IFN- $\alpha 1$  hybrid.

25 The foregoing examples are provided by way of illustration only. One of skill in the art will appreciate that numerous variations on the biological molecules and methods described above may be employed to make and use IFN- $\alpha 21a$ /IFN- $\alpha 2c$  hybrid interferons. We claim all such subject matter that falls within the scope and spirit of the following claims.

Table 1

Interferon	Antiproliferative Activity (ng/ml) <sup>a</sup>			Specific Antiviral Activity (IU/mg)			Binding Activity <sup>125</sup> I-IFN- $\alpha$ 2b (ng/ml) <sup>b</sup> (competes with <sup>125</sup> I-IFN- $\alpha$ 2b)	
	Daudi Cells	WISH Cells	Ratio WISH/Daudi	MDBK Cells	WISH Cells	Ratio MDBK/WISH	Daudi cells	WISH Cells
IFN- $\alpha$ 2c	0.005	80	1.6 x 10 <sup>4</sup>	3.3 x 10 <sup>8</sup>	1.9 x 10 <sup>8</sup>	1.7	35	25
IFN- $\alpha$ 21a	0.0008	95	1.2 x 10 <sup>5</sup>	3.7 x 10 <sup>8</sup>	0.7 x 10 <sup>8</sup>	5.3	>400	>200
HY-1	0.5	110	2.2 x 10 <sup>2</sup>	2.0 x 10 <sup>8</sup>	0.1 x 10 <sup>8</sup>	20.0	>400	>200
HY-2	1.0	>1000	>1 x 10 <sup>3</sup>	3.0 x 10 <sup>8</sup>	0.1 x 10 <sup>8</sup>	30.0	>400	>200
HY-3	0.0001	8	8 x 10 <sup>4</sup>	2.0 x 10 <sup>8</sup>	0.7 x 10 <sup>8</sup>	2.9	30	30
HY-4	1.0	N/D		4.8 x 10 <sup>8</sup>	N/D		N/D	N/D
HY-5	0.03	N/D		3.0 x 10 <sup>8</sup>	N/D		N/D	N/D
SDM-1	0.01	N/D		2.7 x 10 <sup>8</sup>	N/D		N/D	N/D
SDM-2	0.013	N/D		4.6 x 10 <sup>8</sup>	N/D		N/D	N/D
SDM-3	N/D	N/D		4.6 x 10 <sup>8</sup>	N/D		N/D	N/D
SDM-4	N/D	N/D		5.0 x 10 <sup>8</sup>	N/D		N/D	N/D

<sup>a</sup> Concentration of IFN species that inhibits cell growth by 50%<sup>b</sup> Concentration of IFN species that inhibits binding of <sup>125</sup>I-IFN- $\alpha$ 2b by 50%

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## CLAIMS

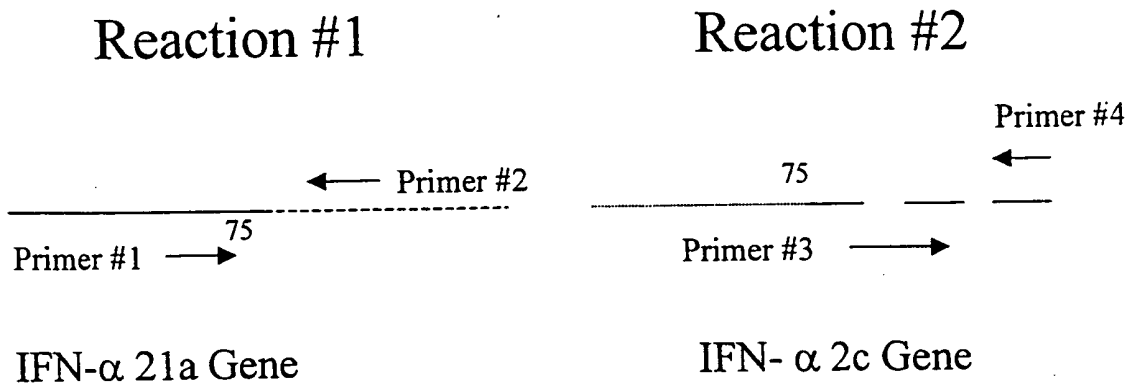
What is claimed is:

1. A purified interferon- $\alpha$  molecule that has interferon- $\alpha$  protein biological activity,  
5 comprising an amino acid sequence from an interferon- $\alpha$ 2c polypeptide, with a mutation of Ser to Tyr at amino acid residue 86 or 90.
2. The purified interferon- $\alpha$  molecule according to claim 1, wherein the interferon- $\alpha$ 2c polypeptide has a mutation of Ser to Tyr at amino acid residues 86 and 90.
3. The purified interferon- $\alpha$  molecule according to claim 1, comprising at least  
10 residues 86 to 90 of the interferon- $\alpha$ 21a polypeptide.
4. The purified interferon- $\alpha$  molecule according to claim 3, comprising at least residues 82 to 95 of the interferon- $\alpha$ 21a polypeptide.
5. The purified interferon- $\alpha$  molecule according to claim 1, wherein the purified interferon- $\alpha$  molecule is a hybrid interferon polypeptide comprising one or more segments of  
15 interferon- $\alpha$ 2c and interferon- $\alpha$ 21a.
6. The hybrid interferon polypeptide according to claim 5, wherein the hybrid comprises at least amino acid residues 86 or 90 of interferon- $\alpha$ 21a.
7. The hybrid interferon- $\alpha$  molecule according to claim 6, comprising an amino acid sequence with a structure M-N-O-P, wherein M comprises about amino acid residues 1-75 of  
20 interferon  $\alpha$ 21a, N comprises about amino acid residues 76 to 81 of interferon- $\alpha$ 2c, O comprises about amino acid residues 82 to 95 of interferon- $\alpha$ 21a, and P comprises about amino acid residues 96 to 165 of interferon- $\alpha$ 2c.
8. A hybrid interferon- $\alpha$  polypeptide, comprising an amino acid sequence selected from the group consisting of:  
25 (a) an amino acid sequence as set forth in SEQ. ID NOS: 9, 11, 13, 30, 32, 34, 36, 38, 40, and 42;  
(b) amino acid sequences with a structure X-A-B, wherein X comprises about amino acid residues 1-75 of an interferon- $\alpha$ , A comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and B comprises about amino acid residues 96-166 of IFN- $\alpha$ 21a;  
30 (c) amino acid sequences with a structure X-A-Y, wherein X comprises about amino acid residues 1-75 of an interferon- $\alpha$ , A comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and Y comprises about amino acid residues 96-165/166 of an interferon- $\alpha$ ; and  
(d) amino acid sequences with a structure V-C-Y, wherein V comprises about amino acid residues 1-81 of an interferon- $\alpha$ , C comprises about amino acid residues 82-95 of IFN- $\alpha$ 2c, and Y comprises about amino acid residues 96-165/166 of an interferon- $\alpha$ ,  
35 wherein the hybrid interferon- $\alpha$  polypeptide has interferon- $\alpha$  protein biological activity.



9. The hybrid interferon- $\alpha$  polypeptide according to claim 8, comprising one or more segments of interferon- $\alpha$ 21a and interferon- $\alpha$ 2c.
10. The hybrid interferon- $\alpha$  polypeptide according to claim 8, comprising an amino acid sequence selected from the group consisting of an amino acid sequence as set forth in SEQ ID NOs: 9, 11, 13, 30, 32, 34, 36, 38, 40, and 42.
11. The hybrid interferon- $\alpha$  polypeptide according to claim 10, wherein the sequence is selected from the group consisting of an amino acid sequence as set forth in SEQ ID NOs: 9, 13, 32, 34, 36, and 38.
12. The hybrid interferon- $\alpha$  polypeptide according to claim 8, comprising the amino acid sequence with a structure X-A-B, wherein X comprises about amino acid residues 1-75 of an interferon- $\alpha$ , A comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and B comprises about amino acid residues 96-166 of IFN- $\alpha$ 21a.
13. The hybrid interferon- $\alpha$  polypeptide according to claim 8, comprising the amino acid sequences with a structure X-A-Y, wherein X comprises about amino acid residues 1-75 of an interferon- $\alpha$ , A comprises about amino acid residues 76-95 of IFN- $\alpha$ 2c, and Y comprises about amino acid residues 96-165/166 of an interferon- $\alpha$ .
14. The hybrid interferon- $\alpha$  polypeptide according to claim 8, comprising amino acid sequences with a structure V-C-Y, wherein V comprises about amino acid residues 1-81 of an interferon- $\alpha$ , C comprises about amino acid residues 82-95 of IFN- $\alpha$ 2c, and Y comprises about amino acid residues 96-165/166 of an interferon- $\alpha$ .
15. A nucleic acid molecule encoding a polypeptide according to claim 8.
16. A recombinant vector comprising the nucleic acid molecule according to claim 15.
17. A cell transformed with the recombinant vector according to claim 16.
18. A pharmaceutical composition comprising:  
a pharmaceutically acceptable vehicle or carrier; and  
at least one hybrid interferon- $\alpha$  polypeptide according to claim 8.
19. A method for treating a patient having a viral disease, comprising administering to said patient a therapeutically effective amount of at least one hybrid interferon- $\alpha$  polypeptide according to claim 8.
20. The method according to claim 19, wherein the administration is by injection.
21. A method for regulating cell growth in a patient, comprising administering to said patient a therapeutically effective amount of at least one hybrid interferon- $\alpha$  polypeptide according to claim 8.
22. The method according to claim 21, wherein the regulated cell growth is tumor cell growth.
23. The method according to claim 21, wherein the administration is by injection.

# PCR #1



↓ PCR Amplification

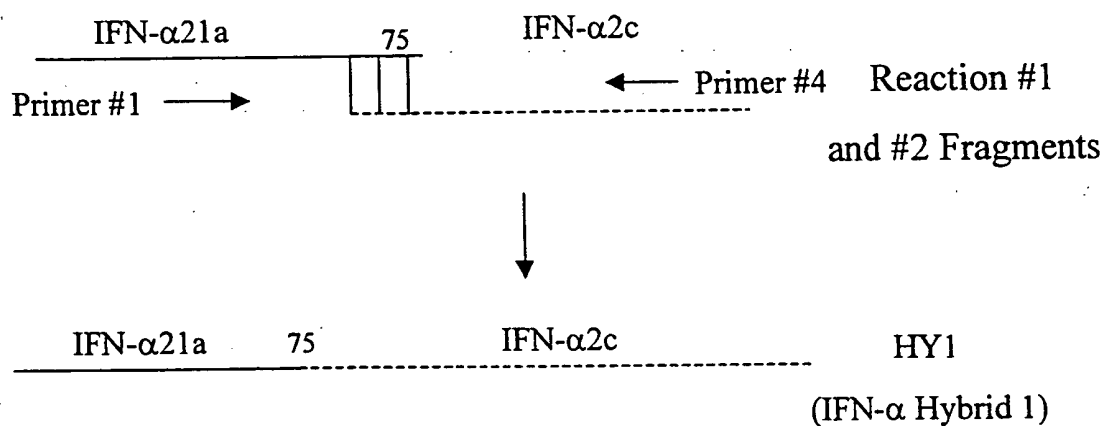
75

α 21a DNA Fragments

75

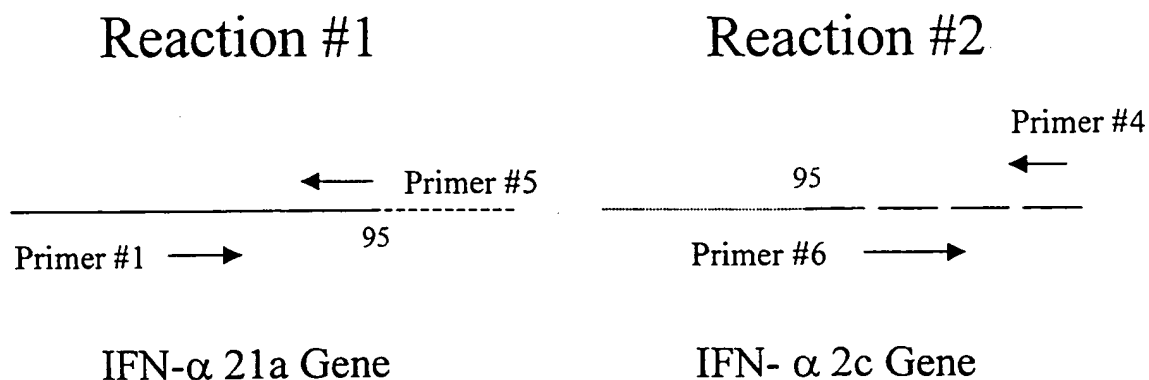
α 2c DNA Fragments

# PCR #2



Construction of Hybrid #1

FIG. 1A

**PCR #1**

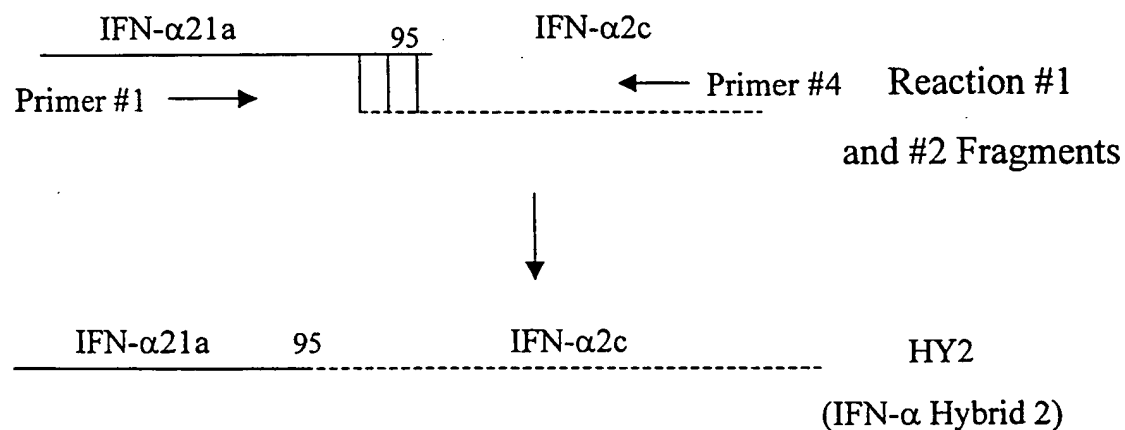
↓ PCR Amplification

95

$\alpha$  21a DNA Fragments

95

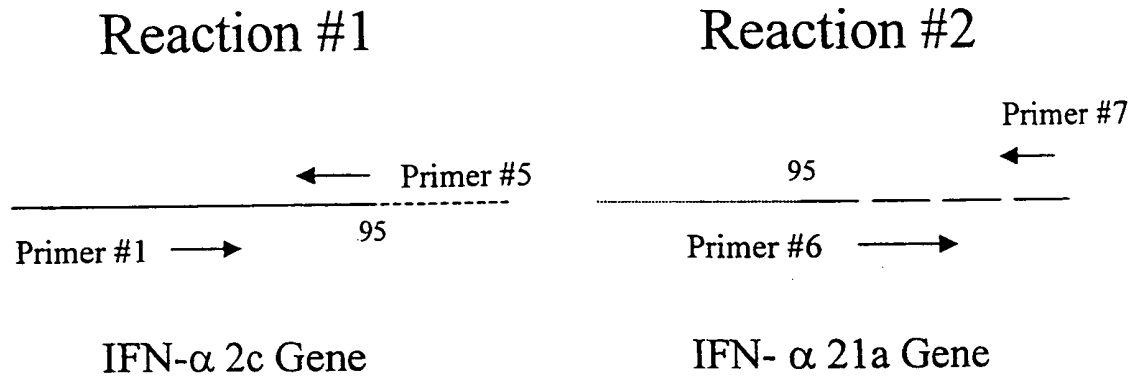
$\alpha$  2c DNA Fragments

**PCR #2**

Construction of Hybrid #2

FIG. 1B

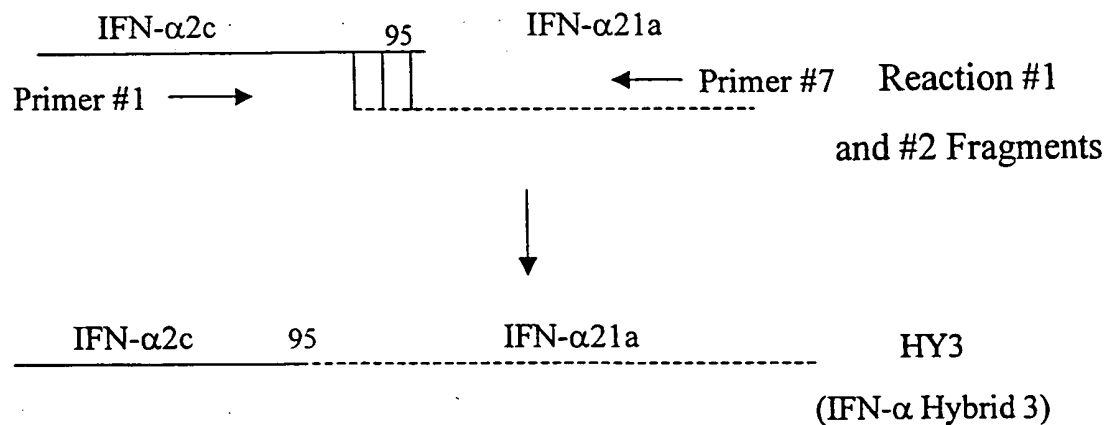
## PCR #1



↓ PCR Amplification



## PCR #2



Construction of Hybrid #3

FIG. 1C

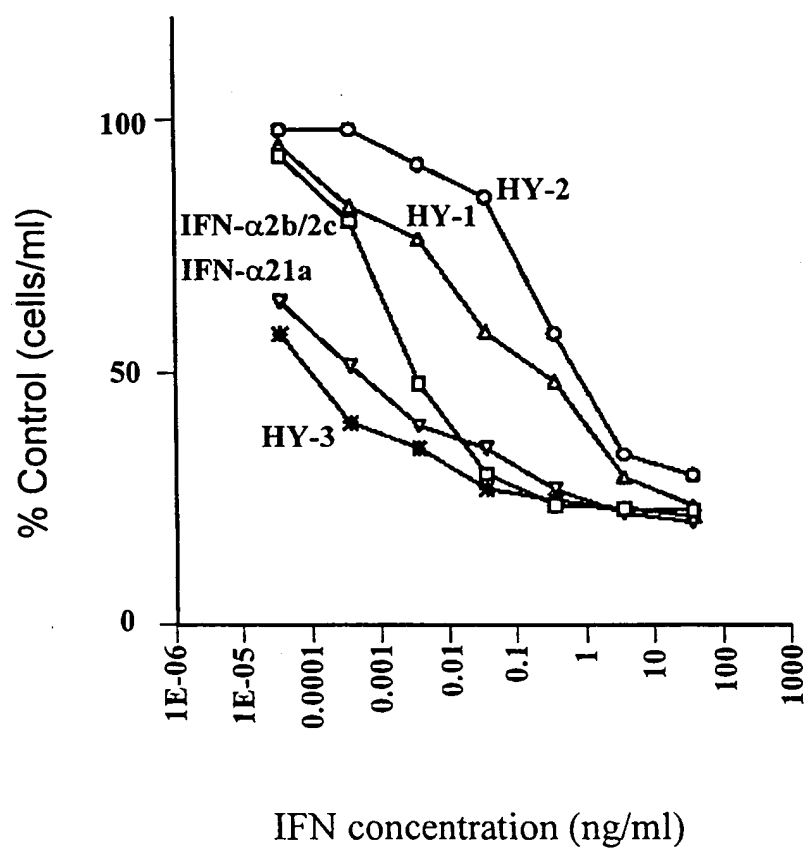


FIG. 2A

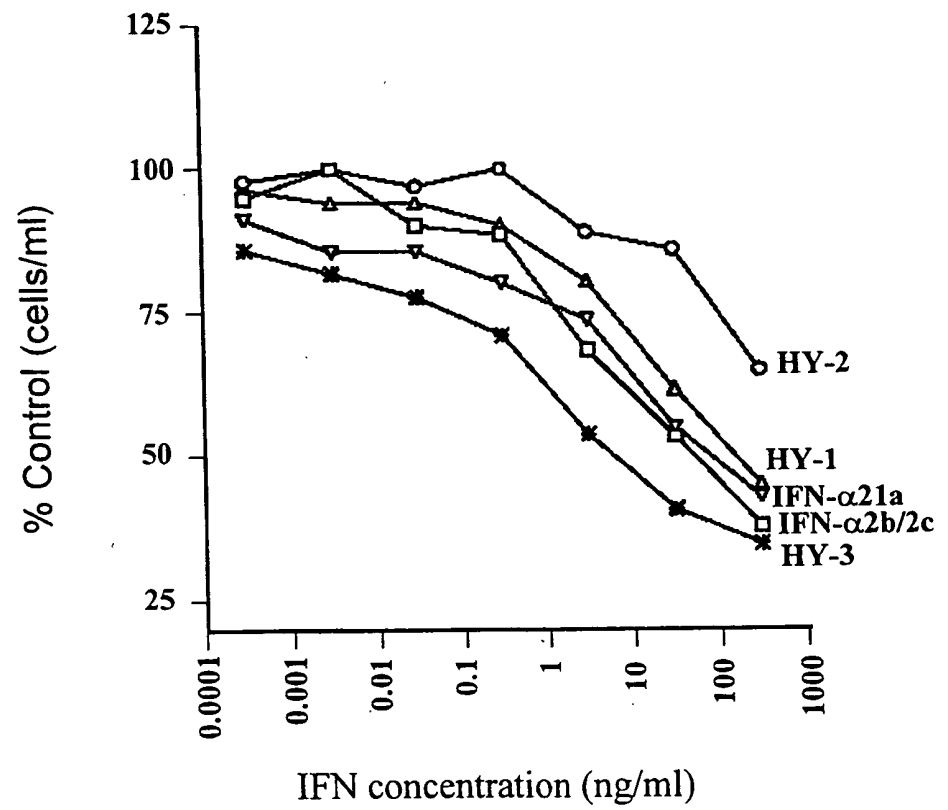


FIG. 2B

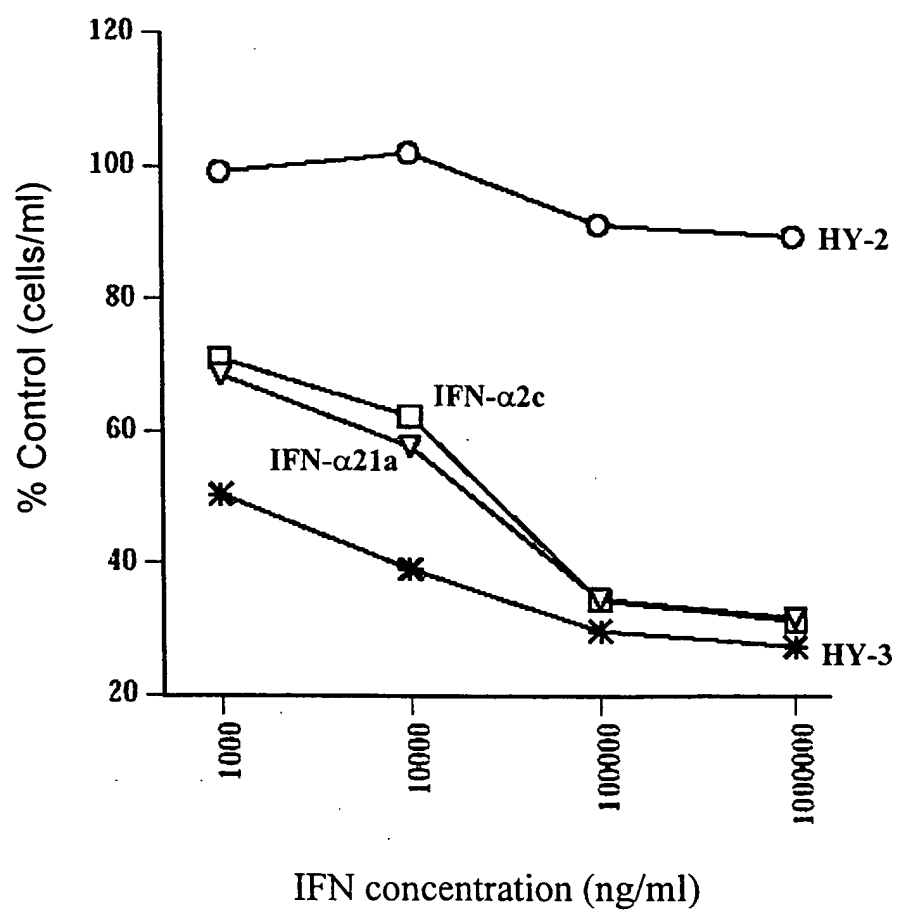


FIG. 2C

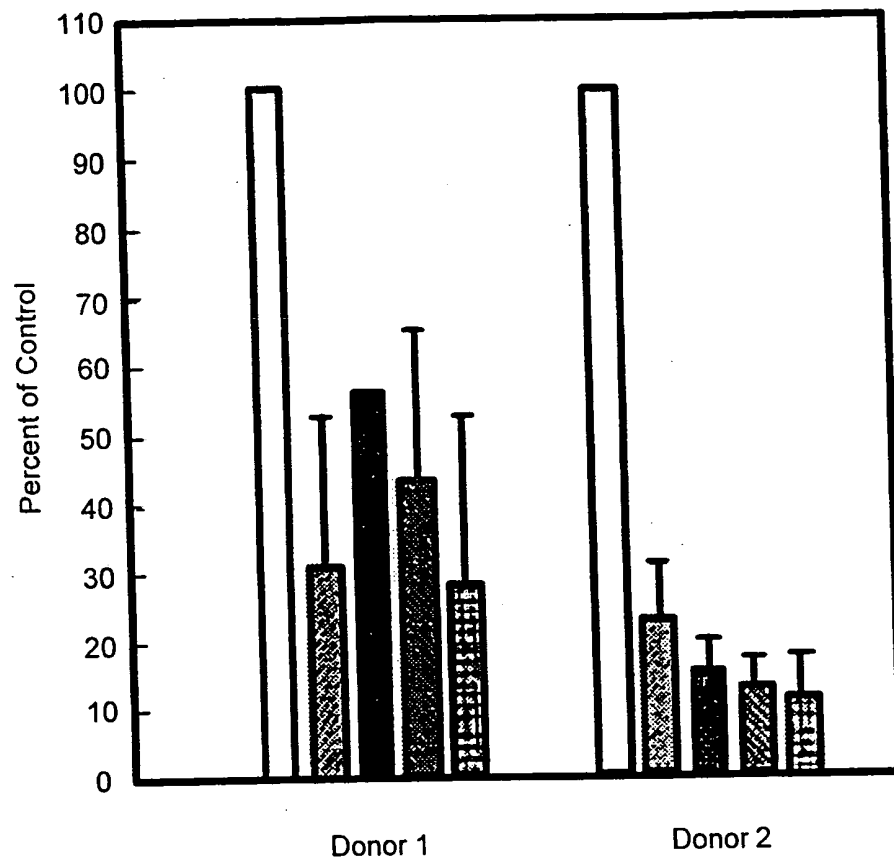


FIG. 3



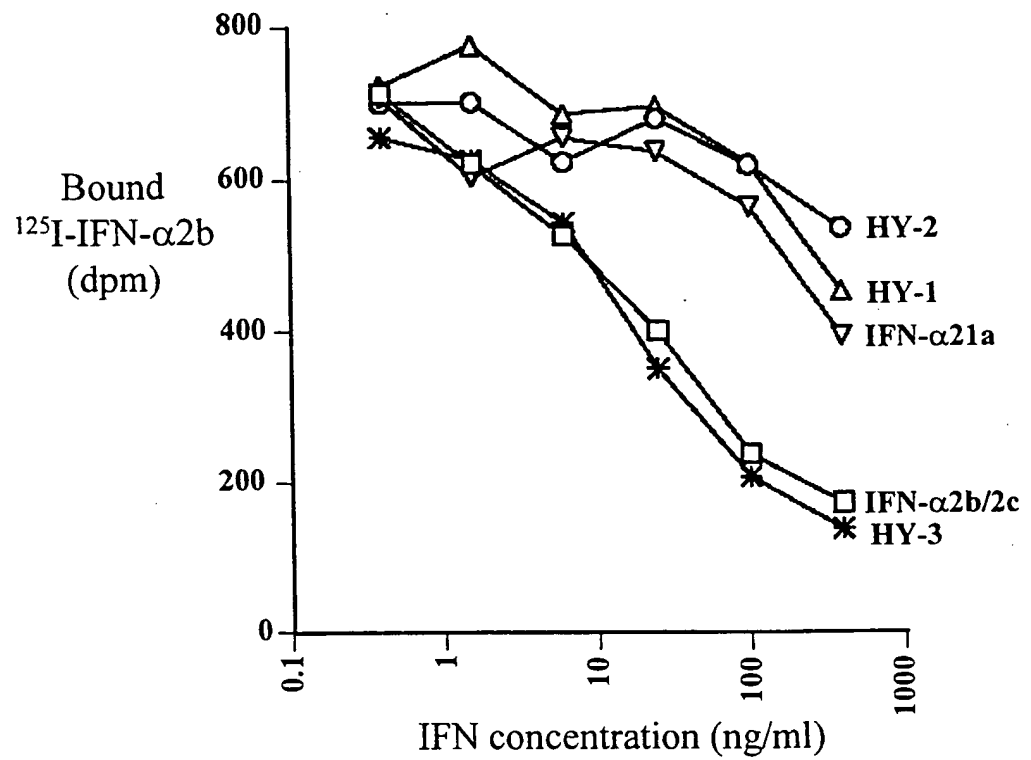


FIG. 4A

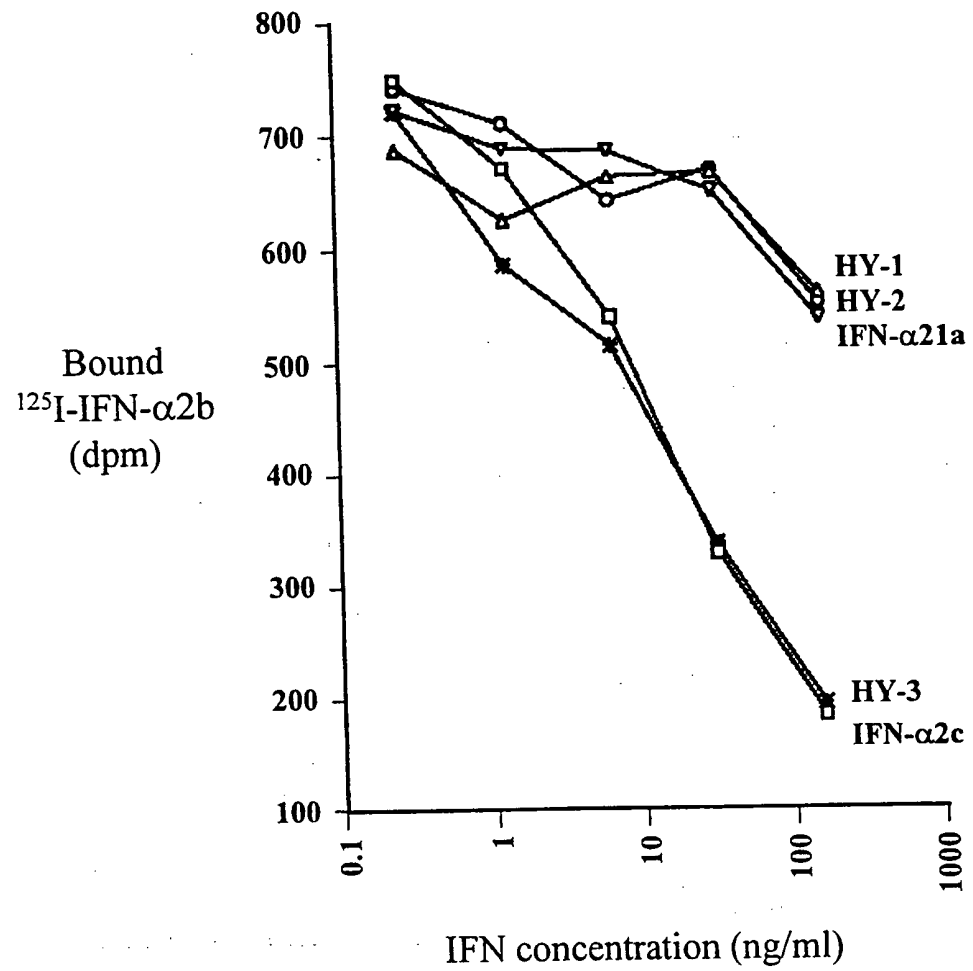


FIG. 4B

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<110> Zoon, et al.

<120> Interferon Alpha Hybrids

<130> 4239-52907

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<150> US 60/094,407

<151> 1998-07-28

<160> 42

<170> PatentIn Ver. 2.0

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<211> 27

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:synthetic  
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27

<210> 2

<211> 27

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:synthetic  
oligonucleotide

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27

<210> 3

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic  
oligonucleotide

<400> 3

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27

<210> 4  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:synthetic  
oligonucleotide

<400> 4  
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36

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<211> 24  
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<223> Description of Artificial Sequence:synthetic  
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<400> 5  
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24

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<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:synthetic  
oligonucleotide

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24

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<223> Description of Artificial Sequence:synthetic  
oligonucleotide

<400> 7  
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36

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:gene fusion

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1) .. (498)

&lt;400&gt; 8

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Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile
  1             5             10             15

ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac 96
Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp
      20             25             30

aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc 144
Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe
      35             40             45

cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc 192
Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr
      50             55             60

ttc aat ctc ttc agc aca aag gac tca tct gct gct tgg gat gag acc 240
Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr
      65             70             75             80

ctc cta gac aaa ttc tac act gaa ctc tac cag cag ctg aat gac ctg 288
Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu
      85             90             95

gaa gcc tgt gtg ata cag ggg gtg ggg gtg aca gag act ccc ctg atg 336
Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met
      100            105            110

aag gag gac tcc att ctg gct gtg agg aaa tac ttc caa aga atc act 384
Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr
      115            120            125

ctc tat ctg aaa gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc 432
Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val
      130            135            140

aga gca gaa atc atg aga tct ttt tct ttg tca aca aac ttg caa gaa 480
Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu
      145            150            155            160

agt tta aga agt aag gaa tg 500
Ser Leu Arg Ser Lys Glu
      165

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 <211> 166  
 <212> PRT  
 <213> Artificial Sequence

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 20 25 30  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
 35 40 45  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr  
 65 70 75 80  
 Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu  
 85 90 95  
 Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met  
 100 105 110  
 Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr  
 115 120 125  
 Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
 130 135 140  
 Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu  
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 Ser Leu Arg Ser Lys Glu  
 165

<210> 10  
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<220>  
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  1             5             10             15

ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac 96
Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp
          20             25             30

aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc 144
Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe
          35             40             45

cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc 192
Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr
          50             55             60

ttc aat ctc ttc agc aca aag gac tca tct gct act tgg gaa cag agc 240
Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Thr Trp Glu Gln Ser
          65             70             75             80

ctc cta gaa aaa ttt tcc act gaa ctt aac cag cag ctg aat gac ctg 288
Leu Leu Glu Lys Phe Ser Thr Glu Leu Asn Gln Gln Leu Asn Asp Leu
          85             90             95

gaa gcc tgt gtg ata cag ggg gtg ggg gtg aca gag act ccc ctg atg 336
Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met
          100             105             110

aag gag gac tcc att ctg gct gtg agg aaa tac ttc caa aga atc act 384
Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr
          115             120             125

ctc tat ctg aaa gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc 432
Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val
          130             135             140

aga gca gaa atc atg aga tct ttt tct ttg tca aca aac ttg caa gaa 480
Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu
          145             150             155             160

agt tta aga agt aag gaa tg 500
Ser Leu Arg Ser Lys Glu
          165

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&lt;210&gt; 11

&lt;211&gt; 166

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;400&gt; 11

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Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile
  1             5             10             15

```

Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
                   20                  25                  30  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
                   35                  40                  45  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
                   50                  55                  60  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Thr Trp Glu Gln Ser  
                   65                  70                  75                  80  
 Leu Leu Glu Lys Phe Ser Thr Glu Leu Asn Gln Gln Leu Asn Asp Leu  
                   85                  90                  95  
 Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met  
                   100                  105                  110  
 Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr  
                   115                  120                  125  
 Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
                   130                  135                  140  
 Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu  
                   145                  150                  155                  160  
 Ser Leu Arg Ser Lys Glu  
                   165

&lt;210&gt; 12

&lt;211&gt; 497

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:gene fusion

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(495)

&lt;400&gt; 12

tgt gat ctg cct caa acc cac agc ctg ggt agc agg agg acc ttg atg 48  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser Arg Arg Thr Leu Met  
                   1                  5                  10                  15  
 ctc ctg gca cag atg agg aga atc tct ctt ttc tcc tgc ttg aag gac 96  
 Leu Leu Ala Gln Met Arg Arg Ile Ser Leu Phe Ser Cys Leu Lys Asp  
                   20                  25                  30  
 aga cgt gac ttt gga ttt ccc cag gag gag ttt ggc aac cag ttc caa 144  
 Arg Arg Asp Phe Gly Phe Pro Gln Glu Glu Phe Gly Asn Gln Phe Gln



35	40	45	
aag gct gaa acc atc cct gtc ctc cat gag atg atc cag cag atc ttc			192
Lys Ala Glu Thr Ile Pro Val Leu His Glu Met Ile Gln Gln Ile Phe			
50	55	60	
aat ctc ttc agc aca aag gac tca tct gct gct tgg gat gag acc ctc			240
Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr Leu			
65	70	75	80
cta gac aaa ttc tac act gaa ctc tac cag cag ctg aat gac ctg gaa			288
Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu Glu			
85	90	95	
gcc tgc gtg ata cag gag gtt ggg gtg gaa gag act ccc ctg atg aat			336
Ala Cys Val Ile Gln Glu Val Gly Val Glu Glu Thr Pro Leu Met Asn			
100	105	110	
gtg gac tcc atc ctg gct gtg aag aaa tac ttc caa aga atc act ctt			384
Val Asp Ser Ile Leu Ala Val Lys Lys Tyr Phe Gln Arg Ile Thr Leu			
115	120	125	
tat ctg aca gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc aga			432
Tyr Leu Thr Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg			
130	135	140	
gca gaa atc atg aga tcc ttc tct tta tca aaa att ttt caa gaa aga			480
Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Lys Ile Phe Gln Glu Arg			
145	150	155	160
tta agg agg aag gaa tg			497
Leu Arg Arg Lys Glu			
165			

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&lt;211&gt; 165

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;400&gt; 13

Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser Arg Arg Thr Leu Met
1 5 10 15

Leu Leu Ala Gln Met Arg Arg Ile Ser Leu Phe Ser Cys Leu Lys Asp
20 25 30

Arg Arg Asp Phe Gly Phe Pro Gln Glu Glu Phe Gly Asn Gln Phe Gln
35 40 45

Lys Ala Glu Thr Ile Pro Val Leu His Glu Met Ile Gln Gln Ile Phe
50 55 60

Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr Leu
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oligonucleotide

<400> 17  
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<210> 18  
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oligonucleotide

<400> 18  
ctgaatgacc tcgaggcctg cgtg 24

<210> 19  
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<220>  
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oligonucleotide

<400> 19  
cacgcaggcc tcgaggcatc tcag 24

<210> 20  
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oligonucleotide

<400> 20  
gaaaaatttt aactgaact t 21

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<212> DNA  
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oligonucleotide

<400> 21  
aagttcagtg taaaattttt c 21

<210> 22  
<211> 21  
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<220>  
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oligonucleotide

<400> 22  
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<210> 23  
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<212> DNA  
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<220>  
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oligonucleotide

<400> 23  
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<210> 24  
<211> 21  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:synthetic  
oligonucleotide

<400> 24  
gacaaattct ccactgaact c 21

<210> 25  
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<223> Description of Artificial Sequence:synthetic  
oligonucleotide

<400> 25

gagttcagtg gagaatttgt c

21

<210> 26

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic  
oligonucleotide

<400> 26

actgaactca accagcagct g

21

<210> 27

<211> 21

<212> DNA

<213> Artificial Sequence

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oligonucleotide

<400> 27

cagctgctgg ttgagttcag t

21

<210> 28

<211> 36

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:synthetic  
oligonucleotide

<400> 28

gagctcgcat gctcatcatt ccttacttct taaact

36

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<212> DNA

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ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac	96
Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp	
20 25 30	
aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc	144
Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe	
35 40 45	
cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc	192
Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr	
50 55 60	
ttc aat ctc ttc agc aca aag gac tca tct gct gct tgg gat gag acc	240
Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr	
65 70 75 80	
ctc cta gaa aaa ttt tcc act gaa ctt aac cag ctg aat gac ctc gag	288
Leu Leu Glu Lys Phe Ser Thr Glu Leu Asn Gln Leu Asn Asp Leu Glu	
85 90 95	
gcc tgt gtg ata cag ggg gtg ggg gtg aca gag act ccc ctg atg aag	336
Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys	
100 105 110	
gag gac tcc att ctg gct gtg agg aaa tac ttc caa aga atc act ctc	384
Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu	
115 120 125	
tat ctg aaa gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc aga	432
Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg	
130 135 140	
gca gaa atc atg aga tct ttt tct ttg tca aca aac ttg caa gaa agt	480
Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser	
145 150 155 160	
tta aga agt aag gaa tg	497
Leu Arg Ser Lys Glu	
165	

&lt;210&gt; 30

&lt;211&gt; 165

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;400&gt; 30

Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile
1 5 10 15

Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
                   20                  25                  30  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
                   35                  40                  45  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
                   50                  55                  60  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr  
                   65                  70                  75                  80  
 Leu Leu Glu Lys Phe Ser Thr Glu Leu Asn Gln Leu Asn Asp Leu Glu  
                   85                  90                  95  
 Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys  
                   100                  105                  110  
 Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu  
                   115                  120                  125  
 Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg  
                   130                  135                  140  
 Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser  
                   145                  150                  155                  160  
 Leu Arg Ser Lys Glu  
                   165

<210> 31  
 <211> 500  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:gene fusion

<220>  
 <221> CDS  
 <222> (1)..(498)

<400> 31  
 tgt gat ctg cct cag acc cac agc ctg ggt aat agg agg gcc ttg ata 48  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
           1                  5                  10                  15  
 ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac 96  
 Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
                   20                  25                  30  
 aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc 144  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
                   35                  40                  45

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<210> 32
<211> 166
<212> PRT
<213> Artificial Sequence
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<400> 32
Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile
  1                      5                      10                      15

Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp
                20                      25                      30

Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe
        35                      40                      45

Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr
        50                      55                      60

Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Thr Trp Glu Gln Ser
        65                      70                      75                      80

Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu

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	85	90	95
Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met			
100	105	110	
Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr			
115	120	125	
Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val			
130	135	140	
Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu			
145	150	155	160
Ser Leu Arg Ser Lys Glu			
165			

<210> 33  
 <211> 500  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:gene fusion

<220>  
 <221> CDS  
 <222> (1)..(498)

<400> 33  
 tgt gat ctg cct cag acc cac agc ctg ggt aat agg agg gcc ttg ata 48  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
 1 5 10 15  
 ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac 96  
 Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
 20 25 30  
 aga cat gac ttt gga ttc ccc caa gag gag ttt gat ggc aac cag ttc 144  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
 35 40 45  
 cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc 192  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60  
 ttc aat ctc ttc agc aca aag gac tca tct gct tgg gat gag acc ctc 240  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Trp Asp Glu Thr Leu  
 65 70 75 80  
 cta gac aaa ttc tac act gaa ctc tac cag cag ctg aat gac ctg atg 288  
 Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu Met  
 85 90 95  
 gaa gcc tgc gtg ata cag gag gtt ggg gtg gaa gag act ccc ctg atg 336

Glu Ala Cys Val Ile Gln Glu Val Gly Val Glu Glu Thr Pro Leu Met  
 100 105 110

aat gtg gac tcc atc ttg gct gtg aag aaa tac ttc caa aga atc act 384  
 Asn Val Asp Ser Ile Leu Ala Val Lys Lys Tyr Phe Gln Arg Ile Thr  
 115 120 125

ctt tat ctg aca gag aag aaa tac agc cct tgt gct tgg gag gtt gtc 432  
 Leu Tyr Leu Thr Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
 130 135 140

aga gca gaa atc atg aga tcc ttc tct tta tca aaa att ttt caa gaa 480  
 Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Lys Ile Phe Gln Glu  
 145 150 155 160

aga tta agg agg aag gaa tg 500  
 Arg Leu Arg Arg Lys Glu  
 165

<210> 34  
 <211> 166  
 <212> PRT  
 <213> Artificial Sequence

<400> 34  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
 1 5 10 15

Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
 20 25 30

Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
 35 40 45

Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60

Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Trp Asp Glu Thr Leu  
 65 70 75 80

Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu Met  
 85 90 95

Glu Ala Cys Val Ile Gln Glu Val Gly Val Glu Glu Thr Pro Leu Met  
 100 105 110

Asn Val Asp Ser Ile Leu Ala Val Lys Lys Tyr Phe Gln Arg Ile Thr  
 115 120 125

Leu Tyr Leu Thr Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
 130 135 140

Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Lys Ile Phe Gln Glu  
 145 150 155 160

Arg Leu Arg Arg Lys Glu  
165

<210> 35

<211> 497

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:gene fusion

<220>

<221> CDS

<222> (1)..(495)

<400> 35

tgt gat ctg cct cag acc cac agc ctg ggt aat agg agg gcc ttg ata	48
Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile	
1 5 10 15	

ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac	96
Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp	
20 25 30	

aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc	144
Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe	
35 40 45	

cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc	192
Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr	
50 55 60	

ttc aat ctc ttc agc aca aag gac tca tct gct gct tgg gat gag acc	240
Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr	
65 70 75 80	

ctc cta gaa aaa ttt tac act gaa ctt aac cag ctg aat gac ctc gag	288
Leu Leu Glu Lys Phe Tyr Thr Glu Leu Asn Gln Leu Asn Asp Leu Glu	
85 90 95	

gcc tgt gtg ata cag ggg gtg ggg gtg aca gag act ccc ctg atg aag	336
Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys	
100 105 110	

gag gac tcc att ctg gct gtg agg aaa tac ttc caa aga atc act ctc	384
Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu	
115 120 125	

tat ctg aaa gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc aga	432
Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg	
130 135 140	

gca gaa atc atg aga tct ttt tct ttg tca aca aac ttg caa gaa agt	480
Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser	
145 150 155 160	

tta aga agt aag gaa tg  
 Leu Arg Ser Lys Glu  
 165

497

<210> 36  
 <211> 165  
 <212> PRT  
 <213> Artificial Sequence

<400> 36  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
 1 5 10 15  
 Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
 20 25 30  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
 35 40 45  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr  
 65 70 75 80  
 Leu Leu Glu Lys Phe Tyr Thr Glu Leu Asn Gln Leu Asn Asp Leu Glu  
 85 90 95  
 Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys  
 100 105 110  
 Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu  
 115 120 125  
 Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg  
 130 135 140  
 Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser  
 145 150 155 160  
 Leu Arg Ser Lys Glu  
 165

<210> 37  
 <211> 497  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:gene fusion  
 <220>  
 <221> CDS

&lt;222&gt; (1) .. (495)

&lt;400&gt; 37

tgt gat ctg cct cag acc cac agc ctg ggt aat agg agg gcc ttg ata 48  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
 1 5 10 15

ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac 96  
 Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
 20 25 30

aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc 144  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
 35 40 45

cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc 192  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60

ttc aat ctc ttc agc aca aag gac tca tct gct gct tgg gat gag acc 240  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr  
 65 70 75 80

ctc cta gaa aaa ttt tcc act gaa ctt tac cag ctg aat gac ctc gag 288  
 Leu Leu Glu Lys Phe Ser Thr Glu Leu Tyr Gln Leu Asn Asp Leu Glu  
 85 90 95

gcc tgt gtg ata cag ggg gtg ggg gtg aca gag act ccc ctg atg aag 336  
 Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys  
 100 105 110

gag gac tcc att ctg gct gtg agg aaa tac ttc caa aga atc act ctc 384  
 Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu  
 115 120 125

tat ctg aaa gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc aga 432  
 Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg  
 130 135 140

gca gaa atc atg aga tct ttt tct ttg tca aca aac ttg caa gaa agt 480  
 Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser  
 145 150 155 160

tta aga agt aag gaa tg 497  
 Leu Arg Ser Lys Glu  
 165

&lt;210&gt; 38

&lt;211&gt; 165

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;400&gt; 38

Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
 1 5 10 15

Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
                     20                    25                    30  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
                     35                    40                    45  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
                     50                    55                    60  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr  
                     65                    70                    75                    80  
 Leu Leu Glu Lys Phe Ser Thr Glu Leu Tyr Gln Leu Asn Asp Leu Glu  
                     85                    90                    95  
 Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys  
                     100                    105                    110  
 Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu  
                     115                    120                    125  
 Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg  
                     130                    135                    140  
 Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser  
                     145                    150                    155                    160  
 Leu Arg Ser Lys Glu  
                     165

<210> 39  
 <211> 500  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:gene fusion

<220>  
 <221> CDS  
 <222> (1)..(498)

<400> 39  
 tgt gat ctg cct cag acc cac agc ctg ggt aat agg agg gcc ttg ata 48  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
                     1                    5                    10                    15  
 ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac 96  
 Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
                     20                    25                    30  
 aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc 144  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
                     35                    40                    45

cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc 192  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60

ttc aat ctc ttc agc aca aag gac tca tct gct act tgg gaa cag agc 240  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Thr Trp Glu Gln Ser  
 65 70 75 80

ctc cta gac aaa ttc tcc act gaa ctc tac cag cag ctg aat gac ctc 288  
 Leu Leu Asp Lys Phe Ser Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu  
 85 90 95

gag gcc tgt gtg ata cag ggg gtg ggg gtg aca gag act ccc ctg atg 336  
 Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met  
 100 105 110

aag gag gac tcc att ctg gct gtg agg aaa tac ttc caa aga atc act 384  
 Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr  
 115 120 125

ctc tat ctg aaa gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc 432  
 Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
 130 135 140

aga gca gaa atc atg aga tct ttt tct ttg tca aca aac ttg caa gaa 480  
 Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu  
 145 150 155 160

agt tta aga agt aag gaa tg 500  
 Ser Leu Arg Ser Lys Glu  
 165

<210> 40  
 <211> 166  
 <212> PRT  
 <213> Artificial Sequence

<400> 40  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
 1 5 10 15

Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
 20 25 30

Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
 35 40 45

Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60

Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Thr Trp Glu Gln Ser  
 65 70 75 80

Leu Leu Asp Lys Phe Ser Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu

85 90 95

Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met  
100 105 110

Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr  
115 120 125

Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
130 135 140

Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu  
145 150 155 160

Ser Leu Arg Ser Lys Glu  
165

<210> 41  
<211> 500  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:gene fusion

<220>  
<221> CDS  
<222> (1)..(498)

<400> 41

tgt gat ctg cct cag acc cac agc ctg ggt aat agg agg gcc ttg ata	48
Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile	
1 5 10 15	
ctc ctg gca caa atg gga aga atc tct cct ttc tcc tgc ctg aag gac	96
Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp	
20 25 30	
aga cat gac ttt gga ttc ccc cag gag gag ttt gat ggc aac cag ttc	144
Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe	
35 40 45	
cag aag gct caa gcc atc tct gtc ctc cat gag atg atc cag cag acc	192
Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr	
50 55 60	
ttc aat ctc ttc agc aca aag gac tca tct gct act tgg gaa cag agc	240
Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Thr Trp Glu Gln Ser	
65 70 75 80	
ctc cta gac aaa ttc tac act gaa ctc aac cag cag ctg aat gac ctc	288
Leu Leu Asp Lys Phe Tyr Thr Glu Leu Asn Gln Gln Leu Asn Asp Leu	
85 90 95	
gag gcc tgt gtg ata cag ggg gtg ggg gtg aca gag act ccc ctg atg	336



Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met  
 100 105 110  
 aag gag gac tcc att ctg gct gtg agg aaa tac ttc caa aga atc act 384  
 Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr  
 115 120 125  
 ctc tat ctg aaa gag aag aaa tac agc cct tgt gcc tgg gag gtt gtc 432  
 Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
 130 135 140  
 aga gca gaa atc atg aga tct ttt tct ttg tca aca aac ttg caa gaa 480  
 Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu  
 145 150 155 160  
 agt tta aga agt aag gaa tg 500  
 Ser Leu Arg Ser Lys Glu  
 165

<210> 42  
 <211> 166  
 <212> PRT  
 <213> Artificial Sequence

<400> 42  
 Cys Asp Leu Pro Gln Thr His Ser Leu Gly Asn Arg Arg Ala Leu Ile  
 1 5 10 15  
 Leu Leu Ala Gln Met Gly Arg Ile Ser Pro Phe Ser Cys Leu Lys Asp  
 20 25 30  
 Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Asp Gly Asn Gln Phe  
 35 40 45  
 Gln Lys Ala Gln Ala Ile Ser Val Leu His Glu Met Ile Gln Gln Thr  
 50 55 60  
 Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Thr Trp Glu Gln Ser  
 65 70 75 80  
 Leu Leu Asp Lys Phe Tyr Thr Glu Leu Asn Gln Gln Leu Asn Asp Leu  
 85 90 95  
 Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met  
 100 105 110  
 Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr  
 115 120 125  
 Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val  
 130 135 140  
 Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu  
 145 150 155 160

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Ser Leu Arg Ser Lys Glu  
165